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Cytoskeletal Innovations in *Giardia lamblia*

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Abstract

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The single-celled eukaryote *Giardia lamblia* is one of the most common gastrointestinal parasites worldwide; infection causes diarrhea and vomiting, which can lead to dehydration and malnutrition. As an early divergent eukaryotic parasite, its cell biology is highly unusual compared to model organisms. *Giardia lamblia*'s actin (*GlActin*) is only 58% identical to the average eukaryotic actin and its genome lacks genes encoding actin interactors in other organisms. Despite this, its actin cytoskeleton retains conserved functions in cell polarity, membrane trafficking, and cytokinesis. To understand how *GlActin* carries out these activities, I used mass spectrometry to identify candidate *GlActin* filament interactors. I categorized the general structure and used fluorescent protein fusions to examine subcellular localizations of 46 putative interactors. The proteins were enriched in the cell cortex, internal membranes, marginal plate, nuclei, flagella, or ventral disc, a microtubule-based organelle that the parasite uses to

attach to its host's intestine. One interactor, denoted Disc and Actin Associated Protein 1 (DAAP1), was enriched in the two areas of the ventral disc that are key for the fluid flow that mediates attachment. I confirmed complex formation between *G/Actin* and DAAP1. I found that *G/Actin* depletion results in severely impaired attachment and defects in disc morphology, while DAAP depletion results in decreased ability to attach and impaired disc seal formation. These studies revealed a role for actin and DAAP1 in ventral disc function. Since attachment is required for infection, targeting attachment could be an avenue for developing new treatments for giardiasis.

TABLE OF CONTENTS

List of Figures	ii
List of Tables	iii
Chapter 1. Introduction	6
1.1 Chapter 1 Figures	13
1.2 Chapter 1 References	17
Chapter 2. Identification of actin filament-associated proteins in <i>Giardia lamblia</i>	24
2.1 Chapter 2 Tables and Figures	50
2.2 Chapter 2 References	101
Chapter 3. Disc and Actin-Associated Protein 1 Influence Host Attachment in the Intestinal Parasite <i>Giardia lamblia</i>	112
3.1 Chapter 3 Tables and Figures	134
3.2 Chapter 3 References	149

LIST OF FIGURES

Figure 1.1. The <i>Giardia</i> life cycle	13
Figure 1.2. Anatomy of a <i>Giardia</i> cell.....	14
Figure 1.3. The structure of the ventral disc	15
Figure 1.4. Stages of <i>Giardia</i> attachment	16
Figure 2.1. Schematic of methods.....	52
Figure 2.2. Localization of 45 <i>GI</i> Actin Filament Interactors.....	53
Figure 2.3. Putative <i>GI</i> Actin interactors with nuclear enrichment	54
Figure 2.4. Putative <i>GI</i> Actin interactors with flagella localization	55
Figure 2.5. Putative <i>GI</i> Actin interactors with marginal plate localization.....	56
Figure 2.6. Putative <i>GI</i> Actin interactors in the endomembrane system.....	57
Figure 2.7. Putative <i>GI</i> Actin interactors in the cortex and plasma membrane.....	58
Figure 2.8. Putative <i>GI</i> Actin Interactors with ventral disc localization.....	59
Figure 2.9. Immunostained <i>GI</i> Actin is localized throughout the cell.....	60
Figure 2.10. Reciprocal co-immunoprecipitation	61
Figure S2.1. Putative <i>GI</i> Actin interactors with non-specific localization or low signal	99
Figure S2.2. Range of mitosome-like localizations in marginal plate proteins	100
Figure 3.1. Depletion of <i>GI</i> Actin affects attachment and ventral disc morphology	134
Figure 3.2. DAAP1 is a <i>GI</i> Actin interactor and a ventral disc-associated protein..	136
Figure 3.3. DAAP1 loading onto the ventral disc requires <i>GI</i> Actin.....	137
Figure 3.4. DAAP1 is stably associated with the ventral disc	138
Figure 3.5. DAAP1-depleted cells have no gross disc morphology defects.....	139
Figure 3.6. DAAP1 depletion results in reduced attachment.....	140
Figure 3.7. DAAP1 has a role in ventral disc seal formation	141
Figure S3.1. <i>GI</i> Actin Depletion by antisense translation-blocking Morpholinos....	142
Figure S3.2. Range of Disc Defect Phenotypes in <i>GI</i> Actin-Depleted Cells	143

LIST OF TABLES

Table 2.1. Proteins identified as putative F-Actin interactors	50
Table S2.1. Previously validated <i>G</i> /Actin interactors identified in this screen	62
Table S2.2. F- <i>G</i> /Actin interactors which have been previously localized	64
Table S2.3. Complete list of proteins identified here as <i>G</i> /Actin interactors	65
Table S2.4. Primer sequences and workflow for tagging each protein.....	92
Table S3.1. Raw number of <i>G</i> /Actin-depleted attachment assay cell counts and statistics	144
Table S3.2. DAAP1-depleted attachment assay cell counts and statistics	145
Table S3.3. Primer and morpholino sequences and workflow	146
Table S3.4. Number of microspheres per cell and statistics	147

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DEDICATION

For every scientist who has ever doubted herself.

Chapter 1. INTRODUCTION

Giardia lamblia is a protozoan parasite that infects approximately 200 million people per year worldwide [1,2], resulting in the gastrointestinal illness giardiasis. Symptoms of giardiasis include nausea, vomiting, and diarrhea, which can lead to malabsorption, dehydration, and malnutrition. The frontline treatment for giardiasis is metronidazole; however, since this drug is a broad spectrum anti-microbial, it can result in microbiota dysbiosis, among other side effects [3,4]. Furthermore, resistance and recurring infections have been reported [5]; these problems are especially concerning in developing countries, where access to clean water is limited [6,7]. *Giardia* is also highly prevalent in countries with clean water access; for instance, *Giardia* infects approximately 1.2 million people per year in the United States [8]. Thus, even with the available drugs and technology, there remains a need for new treatments for giardiasis.

The *Giardia* life cycle is composed of two stages (Figure 1.1. **The *Giardia* life cycle**). The infectious form is an environmentally resistant and metabolically quiescent cyst, which is found in freshwater lakes, ponds, and streams. Upon ingestion by a host, the cyst responds to the acidic environment of the stomach and rapidly undergoes two rounds of cell division, resulting in four trophozoites [9]. These metabolically active trophozoites continue to divide, colonizing the host intestine and causing disease.

Giardia is a diplomonad and a member of the eukaryotic supergroup *Excavata* [10]. Recent analysis suggests that *Excavata* should be dissected into three smaller groups, placing *Giardia* in the *Metamonada* subgroup [11]. Most rooted eukaryotic trees put *Giardia* at the base of the tree [12], meaning that it is one of the earliest branching eukaryotes [13]. Whether due to the unusual environment in which it spends half of its lifecycle or its status as an early-branching

eukaryote, *Giardia* has highly divergent cell biology compared to metazoan model organisms. In many ways, *Giardia*'s systems are reduced in complexity compared with other eukaryotes. For instance, *Giardia* cells lack a Golgi apparatus, peroxisomes, and lysosomes. The functionality of their mitochondria is reduced to simple iron sulfide oxidation [14,15]. Other examples of how *Giardia* has scaled back its cellular architecture include the retromer complex, which lacks sorting nexins [16,17], and the ESCRT-II and -III complexes, which appear to lack VPS36 and VPS60 and IST1, respectively [17,18]. In other ways, *Giardia* cell biology is more complex than that of model organisms: the *Giardia* cell contains two nuclei and their microtubule cytoskeleton, on which their entire mode of parasitism relies, is highly developed. Furthermore, while the *Giardia* genome is reduced compared to other eukaryotes, it has an abundance of NEK kinases [17,19] and proteins containing ankyrin repeat domains [17]. Indeed, *Giardia* boasts the protein with the highest known number of ankyrin repeats [20].

Giardia's divergence is exemplified by its cytoskeleton (Figure 1.2). It has four pairs of flagella: caudal, posteriolateral, ventral, and anterior [21,22]. It also has three unique microtubule organelles. The funis is a rib-like array of microtubules connecting the axonemes responsible for dorsal-ventral flexion. The median body is a microtubule reservoir [23,24]. There is also an adhesive disc, also known as the ventral disc, which provides a mechanical means for the parasite to attach to their host *in vivo*, or to plastic or glass *in vitro* [25].

Despite its minimalist genome of fewer than 5,000 genes [17], *Giardia* has invested heavily in the ventral disc. It is a large structure with over 100 proteins identified thus far as localizing to the structure [26,27]. This investment may reflect the importance of the disc for *Giardia*'s parasitic livelihood. Most of these disc-associated proteins, or DAPs, are annotated in

the genome as “hypothetical proteins,” meaning they have no identifiable domains or known homologues in other organisms

[26,28]. Furthermore, many DAPs appear stably associated with specific subregions within the disc [26]. This suggests that different DAPs and different regions within the disc have different roles for attachment. The most ventral layer of the disc is composed of microtubules, followed by microribbons (Figure 1.3) [29,30]. These flat cytoskeletal structures are composed of giardins, which are apparently *Giardia*-specific intermediate filament proteins [30,31]. These microribbons are joined together by crossbridges, flexible linker proteins which keep the structure of the disc intact [28,31,32].

During cell division, the disc is disassembled and rebuilt into two daughter discs [33]. Once formed, the disc is a highly stable structure. Unlike other microtubule-based structures such as the spindle or flagella, there is no detectable turnover of disc microtubules as assessed by FRAP of disc-associated proteins [27,34,35]. Disc microtubules are not affected by microtubule-depolymerizing drugs, such as nocodazole or colchicine, except when certain DAPs are depleted [27].

This result indicates that disc microtubules are not inherently resistant to these drugs, but are protected from them by DAPs. The disc is nucleated at multiple sites near the anterior of the cell [26]. The sheet of disc microtubules curve around and overlap themselves near the origin of nucleation in a region fittingly named the overlap zone (Figure 1.3). Other notable regions of the disc include the margin of the disc or lateral crest and ventral groove, a hump at the posterior region of the disc where the ventral flagella exit the cell; and the bare area, the center of the disc, devoid of microtubules [26]. Both immunofluorescence and electron microscopy have revealed a dense array of proteins at the lateral crest, which has been proposed to be involved in creating a

seal during attachment [26,28]. The ventral groove has its own subset of proteins, as well as a different curvature from the rest of the disc; it has also been implicated in attachment [31].

The mechanism of disc-based attachment has been controversial, with two different mechanisms previously proposed: hydrodynamic flow and mechanical suction. The flagella are required to initiate, but not maintain, attachment, suggesting that the beating of the ventral flagella pull fluid out from under the disc [33,36]. The current model for disc-based attachment combines the two previous models, and implicates two regions of the disc in regulating attachment. These regions are the ventral groove and the overlap zone, both of which are proposed to act as valves that allow fluid under the disc [25,26,34].

There are four stages of attachment, two early and two late (Figure 1.4). In the first stage, the anterior region of the ventrolateral flange, a thin membrane protrusion, skims along the attachment surface and initiates contact [34,37]. A seal around the edge of the disc is then generated by the lateral crest, corresponding with doming of the disc. Throughout this process, the ventral flagella are beating, drawing fluid under the disc. The anterior valve at the overlap zone then closes as the ventral flagella continue beating, pulling out liquid and creating a vacuum under the disc. Here the posterior region of the ventral plasma membrane, the lateral shield, makes contact with the surface. Finally the posterior valve at the ventral groove closes, creating a seal, and the bare area of the disc descends and contacts the surface.

Attachment is a promising target for drug development. Host attachment is required for maintaining infection and many of the proteins involved in attachment are either highly divergent or unique to *Giardia* [27,28,38]. While some proteins have been identified as contributing to attachment, the mechanism for generating the force on the disc to initiate or maintain attachment is unknown.

The marginal plate, a ridge associated with the axonemes of the anterior flagella (Figure 1.2), is cytoskeletal structure that may be unique to *Giardia* [23,31]. Electron microscopy revealed a dense network of filaments comprising this structure [31], and immunofluorescence microscopy showed an enrichment of *GI*Actin there as well [39,40]. The function of the plate remains poorly understood. It has been hypothesized to be involved in attachment [35,41], but its role in this process has not been studied.

The actin cytoskeleton of *Giardia* is unusual in several ways. *Giardia* actin, or *GI*Actin, is only 58% identical to the average eukaryotic actin [40]; additionally, *Giardia*'s genome lacks all conserved actin interactors [17,42]. *Giardia*'s lack of conventional actin interactors may indicate that it diverged before the appearance of such interactors in later eukaryotic branches. *GI*Actin's divergence may be due to lack of evolutionary constraint provided by the core set of actin-binding proteins. Due this divergence, *GI*Actin is immune to nearly all known actin-targeting drugs, with the exception of jasplakinolide, and is not recognized by live actin markers developed in model organisms. Furthermore, heterologous anti-actin antibodies do not recognize *GI*Actin [40,41].

Alternatively, *Giardia*'s lack of conventional actin interactors could be an example of cryptic genome reduction, particularly since some actin-binding domains have been found in *Crenarchaea* and *Lokiarcheota* [43–45], followed by independent evolution of new types of actin interactors. Despite the extreme differences between actin cytoskeletons of *Giardia* and model organisms, *GI*Actin maintains a conserved and essential role in cytokinesis, membrane trafficking, and cell polarity [40].

Most organisms have multiple isoforms of actin: mammals have six isoforms of actin, including four found in muscle and the two cytoplasmic actins γ - and β -actin [46]. The

expression of the latter two is regulated, and each has its own role: β -actin controls cellular adhesion and contraction, while γ -actin is involved in cell motility. *Giardia* is unusual in that it only has a single actin isoform, which thus must function as a generalist, since *GlActin* has multiple conserved cellular roles [39,40]. Although some other parasites, such as *Toxoplasma gondii*, also have a single isoform [47], others have multiple actins; *Plasmodium falciparum* has two isoforms [48] and *Trypanosoma cruzi* has four [49]. *Giardia*'s closest relative, the salmon parasite *Spironucleus salmonicidae*, also has a single isoform [50]. Notably, the *Spironucleus* genome contains six actin-binding proteins [39,50].

While the dynamics of filament formation of *Giardia* actin are unknown, other parasite actins may give a clue to its behavior. The two most well-studied parasite actins are those of the apicomplexans *Plasmodium falciparum* and *Toxoplasma gondii*, both of which use an actin-based gliding motility [51,52]. *Toxoplasma gondii* actin (*TgActin*) has been the subject of much controversy. While there is evidence of *TgActin* forming short filaments *in vitro* [53], it has also been shown to form long filaments *in vivo* [54,55]. Furthermore, *TgActin* is believed to undergo isodesmic polymerization, meaning the subunit on-rate and off-rate are equal [53]. This would make *TgActin* the only actin with no critical concentration. In contrast, *PfActin*, has a critical concentration roughly ten times that of skeletal actin and while *PfActin* is capable of forming long filaments, they are highly unstable upon ATP hydrolysis [56]. Notably, while both *Toxoplasma* and *Plasmodium* lack the full set of canonical actin-binding proteins [57], they do contain more than *Giardia*.

Here I identify and localize 46 novel *GlActin* filament interactors, which are enriched in the following categories: cell cortex and plasma membrane, internal membranes, marginal plate,

flagella, nuclei, or ventral disc. I then focus on one of these interactors from the latter category and investigate the role of it, and *GlActin*, in ventral disc-based attachment.

1.1 CHAPTER 1 FIGURES

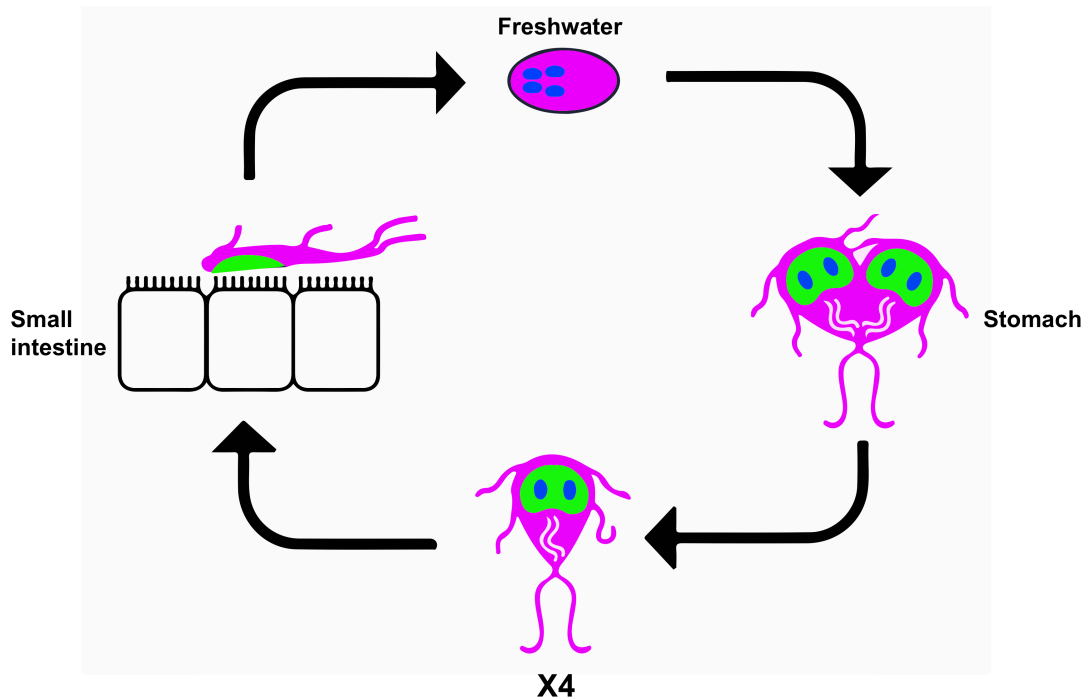


Figure 1.1. The *Giardia* life cycle

There are two stages in the *Giardia* life cycle: the infectious cyst (top), which is found in lakes and streams and the proliferative trophozoite (bottom). Upon ingestion by an animal, the cyst responds to the environment of the stomach and rapidly undergoes two rounds of cell division, resulting in four trophozoites, which then attach to the epithelial cells of the small intestine.

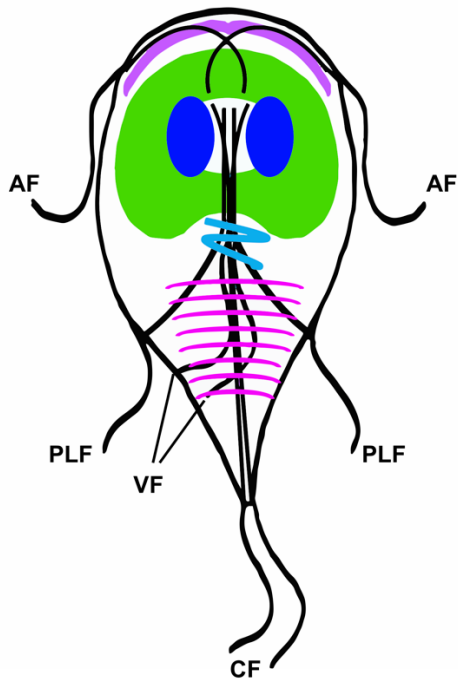


Figure 1.2. Anatomy of a *Giardia* cell

In addition to its two nuclei (royal blue), *Giardia*'s divergence is exemplified in its cytoskeleton. *Giardia* cells have four pairs of flagella: the anterior (af), ventral (vf), posterolateral (plf), and caudal (cf). The ventral disc (green) is a microtubule-based organelle specialized attachment. Also shown are the median body, a microtubule reservoir (light blue) and the funis (magenta), a rib-like microtubule array connected to the central axonemes. The marginal plate (purple) is a structure of unknown function associated with the axonemes of the anterior flagella.

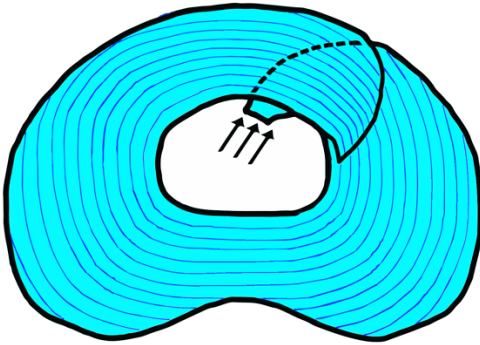
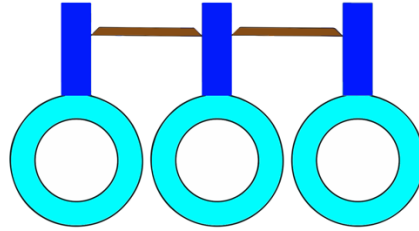
A**B**

Figure 1.3. The structure of the ventral disc

A.) The ventral disc is composed of a sheet of stable microtubules which wraps around and overlaps itself. B.) A view of the disc from the arrows shown in A.) Microtubules are shown in cyan, microribbons in blue, and crossbridges in brown.

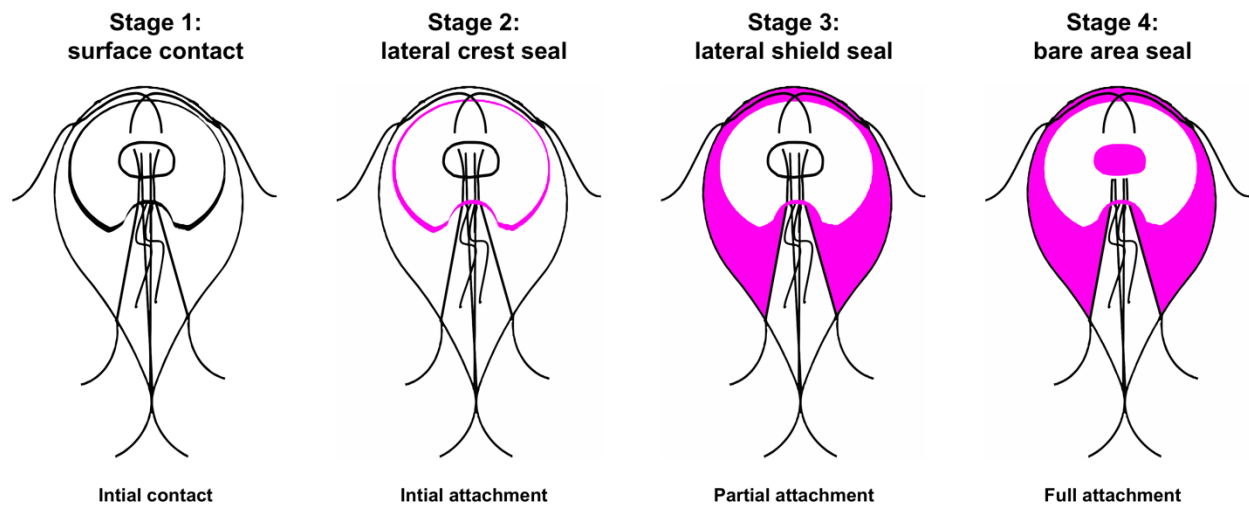


Figure 1.4. Stages of *Giardia* attachment

Giardia cells attach to a surface in four stages. In stage 1, unattached cells make contact with the attachment surface. A seal forms around the lateral crest in stage 2. The lateral shield makes contact with the surface in stage 3. The cell is fully attached in stage 4 as a vacuum is generated under the disc, resulting in contact between the bare area and the attachment surface.

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Chapter 2. IDENTIFICATION OF ACTIN FILAMENT-ASSOCIATED PROTEINS IN *GIARDIA LAMBLIA*

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Abstract

The deep-branching protozoan parasite *Giardia lamblia* is the causative agent of the intestinal disease giardiasis. Consistent with its proposed evolutionary position, many pathways are minimalistic or divergent, including its actin cytoskeleton. *Giardia* is the only eukaryote known to lack all canonical actin-binding proteins; these proteins perform such functions as nucleating or severing filaments and sequestering monomers, among others. Previously, our lab identified non-canonical *Giardia lamblia* actin (*GlActin*) interactors; however, these proteins appeared to interact only with monomeric or globular actin (G-actin), rather than filamentous actin (F-actin). To identify F-actin interactors, we used a chemical crosslinker to preserve native interactions, followed by an anti-*GlActin* antibody, Protein A affinity chromatography, and liquid chromatography coupled to mass spectrometry. We found 46 putative actin interactors enriched in the conditions favoring F-actin. Data are available via ProteomeXchange with identifier PXD026067. None of the proteins identified contain known actin-interacting motifs, and many lacked conserved domains. Each potential interactor was then tagged with the fluorescent protein mNeonGreen and visualized in live cells. We categorized the proteins based on their primary localization; localizations included ventral disc, marginal plate, nuclei, flagella, plasma membrane, and internal membranes. One protein from each of the six categories identified was

co-localized with *GI*Actin using immunofluorescence microscopy. We also co-immunoprecipitated one protein from each category and confirmed three of the six potential interactions. Most of the localization patterns are consistent with previously demonstrated *GI*Actin functions, but the ventral disc represents a new category of actin interactor localization. These results suggest a role for *GI*Actin in ventral disc function, which has previously been controversial.

Importance

The single-celled eukaryote *Giardia lamblia* is an intestinal parasite that colonizes the small intestine and causes diarrhea and vomiting, which can lead to dehydration and malnutrition. While treatments for *Giardia* exist, drug resistance is becoming more common, resulting in a need for new treatments. *Giardia* and human systems are highly dissimilar, thus drugs specifically tailored to *Giardia* proteins would be less likely to have side effects. *Giardia* actin (*GI*Actin) has a conserved role in *Giardia* cells, despite being a highly divergent protein with none of the conserved regulators found in model organisms. Here we identify and localize 46 interactors of polymerized actin. These putative interactors localize to a number of places in the cell, underlining *GI*Actin's importance in multiple cellular processes. Surprisingly, eight of these proteins localize to the ventral disc, *Giardia*'s host attachment organelle. Since host attachment is required for infection, proteins involved in this process are an appealing target for new drugs.

Introduction

Actin is a highly conserved filament-forming protein with essential roles in all eukaryotes that include signaling, motility, membrane trafficking, cell polarity, and cytokinesis [1]. Both

actin monomers (globular or G-actin) and polymerized actin (filamentous or F-actin) have essential functions in the cell; therefore, the balance between the two forms has a regulatory role in addition to a structural one [2]. Eukaryotes throughout the evolutionary tree possess regulators which spatially and temporally control filament formation and depolymerization; their functions include monomer sequestration, filament nucleation or elongation, severing, capping, and crosslinking of filaments [1]. Other actin interactors fulfill such roles as linking to organelles and the plasma membrane or moving cargo and generating contractile forces.

The protozoan parasite *Giardia lamblia* is the only eukaryote known to lack the canonical actin-binding proteins [3]. Due to the lack of conserved interactors that constrain actin evolution, *Giardia* possesses the most divergent actin identified to date, and is only 58% identical to the average eukaryotic actin; in contrast, *S. cerevisiae* actin and human skeletal actin are 87% identical [4,5]. However, *Giardia* actin (*GIActin*) retains conserved roles in many cellular processes, including membrane trafficking, cell polarity, and cytokinesis [6,7]. These conserved roles indicate the presence of non-canonical interactors in the proteome [8]. Giardiasis has been designated a neglected disease by the World Health Organization, and giardiasis results in millions of cases of diarrheal disease worldwide each year [9]. Hence, the study of the cellular role of *GIActin*, as well as its extreme divergence, could make it and its interactors potential drug targets.

Our lab previously identified actin-associated proteins in *Giardia* [10]; that study focused on proteins with conserved, identifiable domains whose actin-binding function either may have been overlooked or may not exist in other organisms. Those interactors included microtubule and flagella-related proteins such as p28 and centrin, the chaperone HSP70, the DNA helicase TIP49, the nuclear ARP7, the atypical MAP kinase ERK7, and the regulatory protein 14-3-3.

Much of the *Giardia* genome contains genes annotated as “hypothetical,” as they do not contain any known domains and/or are unique to *Giardia*. Our previous study identified novel actin-binding partners in *Giardia* [10] but did not examine these particular proteins further. This study also used a twin-strep tag to affinity purify *GI*Actin and did not utilize buffer conditions that stabilize actin filaments; furthermore, the punctate localization of the interactors described were consistent with that of monomeric actin. Therefore, it is likely that our earlier work may have missed proteins that bind exclusively to filamentous actin and additional actin interactors in *Giardia* remain to be discovered.

Here, we used a different approach to discover novel *GI*Actin interactors for the purpose of identifying those which bind F-actin. Using a custom anti-*GI*Actin antibody, Protein A affinity chromatography, and liquid chromatography tandem mass spectrometry (LC -MS/MS), we identified previously unknown candidate *GI*Actin interactors. We categorized a subgroup of these putative interactors based on their localization: marginal plate, flagella, ventral disc, nuclei, membrane, and nonspecific. Notably, no previous *GI*Actin interactors have been localized to the marginal plate or the ventral disc, suggesting that there may be previously unappreciated roles for *GI*Actin in these cellular structures.

Results

Identification of Non-Canonical *GI*Actin Interactors

We used a biochemical approach to identify *GI*Actin-binding proteins, utilizing a chemical crosslinker, anti-*GI*Actin antibodies, and Protein A beads. For our purification scheme, we used buffers developed for canonical actin purification; whether these buffers are optimal for *GI*Actin remains untested. Therefore, we also treated the cells with the cleavable chemical

crosslinker dithiobis (succinimidyl propionate) (DSP) to ensure the stabilization of native interactions before cell lysis. This molecule reacts with both lysine side chains and the amino terminus of peptides, linking amine groups within 12 angstroms of one another.

In order to differentiate G-actin from F-actin interactors, we lysed wild type trophozoites under three different conditions using two different buffers. Compared with low salt G-Buffer, our F-buffer contains KCl, MgCl₂, EGTA, and Imidazole, which promote and stabilize filamentous actin [6,11]. The three different conditions were: A) in G-buffer, expected to favor monomeric actin, followed by DSP treatment; B) in F-buffer, expected to favor filamentous actin, followed by DSP treatment, or C) pre-treated with DSP before lysis in F-buffer, expected to stabilize native interactions (Figure 2.1). We then purified *G*/Actin using an anti-*G*/Actin antibody and Protein A affinity chromatography, and subsequently identified the interactors by LC-MS/MS.

Proteins were classified as potential F-actin interactors if they were enriched in either the DSP pre-treated or F-actin buffer conditions in at least two out of three replicates as compared to the G-actin condition. Notably, proteins that had been previously identified as *G*/Actin interactors, including 14-3-3, p28, GASP-180, and others, were also found in this screen consistent with our expectations for these new methods (Table S2.2). Proteins enriched in the F-conditions but that are commonly found as contaminants such as ribosomal and proteasomal proteins [12], were omitted from our analysis. We also omitted metabolic proteins, to focus on candidates that might directly regulate actin organization, and VSPs (variant-specific surface proteins involved in antigenic variation [13,14]. After exclusion criteria were applied, a total of 46 proteins remained (Table 2.1). Many of these proteins were annotated as “hypothetical proteins,” meaning they lack homologues or known protein domains. Each candidate protein was

tagged with the fluorescent protein mNeonGreen and localized in live and/or fixed cells. Five proteins had non-specific localization or low signal (Figure S2.11, Table 2.1. **Proteins identified as putative F-Actin interactors**); the others were grouped into the categories discussed below based on their localization (Figure 2.2). Despite multiple attempts, we were unable to transform GL50803_21423 (beta adaptin) and thus discarded from further analysis.

***Gl*Actin Interactors in the Nuclei**

Giardia has two transcriptionally active nuclei, to which *Gl*Actin localizes [6]. Previous studies identified conserved *Gl*Actin interactors in the nuclei and treatments altering the phosphorylation state of *Gl*Actin affected nuclear size [10,15]. In model organisms, actin has multiple roles in the nucleus, including chromatin remodeling and regulating transcription [16].

Our screen discovered six nuclear-enriched proteins, each with a distinct sublocalization based on expression of mNeonGreen protein fusions (Figure 2.3). GL50803_5238 (sigma adaptin, a component of the AP-2 complex) appeared in puncta within the nuclei and in association with the cell cortex. GL50803_102813 (ankyrin repeat protein), a protein upregulated during early encystation [17], also appeared in the cytoplasm, with some large puncta, as well as a slight enrichment in the marginal plate. The protein appears somewhat more concentrated towards the anterior of the nuclei. GL50803_GL14299 (hypothetical) appeared to be associated with the nuclear envelope in puncta. The perinuclear localization of GL50803_101212 (hypothetical) may represent the perinuclear endoplasmic reticulum. GL50803_33989 (hypothetical) was previously tagged with a C-terminal GFP tag and formed inclusion bodies in cells [18,19], so we tagged this protein N-terminally under its native promoter. Under these conditions, GL50803_33989 localized to the nuclei, forming structures which may be

filamentous. In contrast, GL50803_14213 (coiled coil domain) has no puncta or other sub-pattern within the nuclei.

***GI*Actin Interactors in the Flagella**

Giardia's four sets of flagella have different cellular roles and undergo a complex developmental cycle [20,21]. Actin is a key component of flagella; in *Chlamydomonas*, six of the seven inner dynein arms form a complex with actin [22]. Additionally, actin is an important regulator of intraflagella transport and the regulation of flagella length [23]. Anti-*GI*Actin immunostaining showed its clear enrichment within the flagella, and as a helix that surrounds the caudal flagella, similar to actin organization of the sperm midpiece [24]. Our previous study identified six axonemal dynein heavy chains in addition to p28 as *GI*Actin interactors [10]. Thus, the identification of six flagellar-enriched proteins in this screen is consistent with previous knowledge (Figure 2.4). Three of these candidate *GI*Actin interactors are dynein light chains; while all appeared in the cytoplasm at low levels, each had a unique flagellar enrichment pattern. GL50803_9848 appeared primarily associated with the cytoplasmic portions of axonemes, while GL50803_15124 was enriched in the entirety of the flagella axonemes. GL50803_4463 had similar axonemal enrichment, and additionally appeared in patches throughout the cell in low levels. GL50803_15097 (alpha-14 giardin) enrichment was less intense in the anterior flagella than the other three flagella pairs. GL50803_33685 (armadillo repeat) appeared throughout the flagella but was particularly enriched in the axonemes. In contrast, GL50803_13774 (hypothetical) appeared only in the flagellar pores, the structures where the flagella exit the cell.

***GL*Actin Interactors in the Marginal Plate**

Seven proteins in our screen were enriched in the marginal plate (Figure 2.5), a lattice-like structure at the anterior of the cell which is associated with the axonemes of the anterior flagella [25]. Ultra-high resolution scanning electron microscopy has shown flexible filaments at the plate [26,27], and our immunofluorescence revealed enriched *GL*Actin in the region [10,27]. Little else is known about the function or composition of the marginal plate; it has been suggested to have a role in attachment, but this has remained an unexplored area of research [6,8,28]

In addition to their presence in the marginal plate, GL50803_115478 (ankyrin repeat), , GL50803_4383 (ankyrin repeat protein), and GL50803_17153 (alpha-11 giardin) also appeared as puncta or linear stretches near the axonemes, a localization reminiscent of the mitochondria-like mitosomes [29,30]. These puncta were not present in all cells, and were variable in number (Figure S2.12). We observed 58.33% (n=48) of GL50803_115478 cells contained puncta, which ranged from 1 to 3, whereas 34.6% (n=52) of GL50803_17153 cells contained puncta, ranging from 1 to 4. All observed GL50803_4383 cells (n=50) contained puncta, ranging from 2 to 5.

Notably, three marginal plate proteins also appeared in microtubule-based structures. GL50803_17153 (alpha-11 giardin) appeared faintly in the disc (Figure S2.12), GL50803_8854 (hypothetical) also localized to the flagellar pores, and GL50803_5800 (hypothetical) was slightly enriched in the axonemes. Although enriched in the marginal plate, GL50803_5800, GL50803_16522, and GL50803_9030 (ankyrin repeat) were also distributed throughout the cytoplasm; GL50803_16522 was also slightly enriched in the nuclei. GL50803_9030 also appeared to be more concentrated in the anterior of the cell in the ventrolateral flange and in the lateral shield, the ventral region of the plasma membrane. Both structures make contact with the

surface of substrates during attachment [31]. Notably, none of the proteins identified in the marginal plate localized exclusively to the plate, suggesting these proteins may have multiple roles.

***GI*Actin Interactors in Internal Membranes**

Giardia has a defined endomembrane system and vesicle trafficking pathways even though it lacks many organelles such as the Golgi, lysosomes, and peroxisomes. *GI*Actin is important for receptor-mediated endocytosis, membrane remodeling during abscission, and the trafficking of cyst wall protein during encystation [6,8,32].

Four of our newly identified *GI*Actin interactors have localization consistent with membrane-bound compartments (Figure 2.6). Apart from the previously mentioned GL50803_101212 (Figure 2.3), which may be in the perinuclear ER, two proteins localize to an internal structure that is likely the endoplasmic reticulum: GL50803_13930 (Arf3) and GL50803_22855 (hypothetical). GL50803_22855 (hypothetical) appeared to be broadly distributed, whereas GL50803_13930 (Arf3) localization to puncta is consistent with enrichment at the ER exit sites [33]. GL50803_9861 (hypothetical) had a punctate localization which could represent small vesicles. Intriguingly, GL50803_12999 (hypothetical) localized to a previously undescribed organelle, as well as puncta that could be peripheral vacuoles and the ER.

***GI*Actin Interactors at the Cortex and Plasma Membrane**

We identified ten putative interactors which localized to the cell cortex, composing the largest group in the screen. This is consistent with *GI*Actin having roles in regulating cell shape, cell polarity, and membrane trafficking (Figure 2.7). Despite a shared generalized localization,

each putative interactor had its own individual pattern. GL50803_15591 (coil coil protein), GL50803_4259 (clathrin light chain), GL50803_103855 (VPS29), GL50803_23833 (VPS35), GL50803_8560 (hypothetical), GL50803_5810 (hypothetical), and GL50803_11684 (leucine rich repeat protein) all had a punctate localization at the cell cortex, likely in association with the plasma membrane. The internal membrane localization of GL50803_8044 (7 transmembrane domain) localizes to both the plasma membrane and what is likely the ER, as transmembrane proteins are necessarily trafficked through this organelle. Both GL50803_14551 (alpha-6 giardin) and GL50803_17255 (hypothetical) are present in the ventrolateral flange, a thin membranous structure that protrudes from the plasma membrane and contributes to parasite attachment [34,35].

***Gl*Actin Interactors in the Ventral Disc**

The ventral adhesive disc is a microtubule-based structure unique to *Giardia*, which allows cells to attach to the small intestine of their host, or to the side of a culture tube *in vitro*. The dome-shaped disc is composed of roughly 100 microtubules which form a stable sheet that wraps into a spiral and overlaps itself [36]. In the center of the disc is the bare area, a region devoid of microtubules but containing membrane-bound vesicles involved in membrane trafficking [31].

Eight putative *Gl*Actin interactors are enriched in the disc, representing the second largest group of putative interactors. GL50803_8726, GL50803_16844, GL50803_4239, and GL50803_17230 had not been localized when we started this work, but have since been identified as disc-associated proteins (DAPs) [19], they are included here because they were

recovered as putative actin binding proteins. GL50803_27925, GL50803_113622, GL50803_24451, and GL50803_11354 are new DAPs.

The localizations within the ventral disc varied widely among the proteins (Figure 2.8). Four of these proteins have no recognized domains, including GL50803_8726 (hypothetical), which localized to the entirety of the ventral disc in addition to the median body, a reservoir of microtubules supporting rapid disc assembly during mitosis [37]. GL50803_16844 (hypothetical), GL50803_4239 (hypothetical), and GL50803_1720 (gamma-giardin) were also apparent in the entirety of the disc, but enriched in specific subregions. GL50803_11354 (hypothetical) and GL50803_24451 (FGF domain) had less distinct disc enrichment. The remaining two disc-localized proteins have ankyrin-repeat domains, and coincidentally had inverse patterns of localization: while GL50803_27925 appeared widely distributed in the disc except the ventral groove, GL50803_113622 localized to only the ventral groove.

Co-localization of Candidate Interactors with *GlActin*

We selected one protein per category of localization to ask if the selected proteins co-localized with actin (Figure 2.9). As there is no *GlActin* marker available for live imaging, we performed immunofluorescence assays using an anti-*GlActin* antibody of fixed cells. The conventional F-actin probe phalloidin does not bind to *GlActin* [6], so polymerized *GlActin* is not easily distinguished from pools of monomeric *GlActin*. As *GlActin* is widely distributed throughout the cell, co-localization with other proteins is not easily discerned.

To increase the sensitivity of detection of coincident signals, we used the JACoP plugin from ImageJ to evaluate co-localization of three image stacks of each protein. Based on Pearson's Correlation Coefficients (PCC), the most prominent co-localization between *GlActin* and its

putative interactors can be seen in the internal membrane protein GL50803_22855 and GL50803_13774, which localizes to the flagellar pores. The nuclear-enriched GL50803_102813 and the marginal plate protein GL50803_9030 also had PCCs > 0.5, indicating co-localization.

In order to validate association with *GlActin* for a subset the identified proteins, we made 3xHA constructs to perform co-immunoprecipitations, following the same lysis and cross-linking procedure used above. We chose one protein per category based on its presence in all three replicates of our proteomics screen. As none of the proteins from the marginal plate category were found in all three replicates, we chose GL50803_8854 based on its high expression and presence at both the marginal plate and flagellar pores, structures where *GlActin* is enriched. We were able to confirm complex formation between *GlActin* and GL50803_27925 (disc), GL50803_8854 (marginal plate), and VPS29 (plasma membrane). Association between *GlActin* and GL50803_12999 (internal membranes) was ambiguous (Figure 2.10), possibly due to the latter's association with membranes and/or low expression. The nuclear interactor GL50803_33989 did not express at high enough levels to be visible on a Western blotting (data not shown). Our co-immunoprecipitation results confirm that we have identified at least three *GlActin*-associated proteins, increasing our confidence in the data presented here. It should be noted, however, that despite its co-localization with *GlActin*, the flagellar pore protein GL50803_13774 did not co-immunoprecipitate with *GlActin*. This does not necessarily indicate that they do not interact; the 3xHA tag could have interfered with the interaction. Another possibility is that the interaction is too weak to be detected by Western blot assay, since LC-MS/MS is a more sensitive technique.

Discussion

We performed LC-MS/MS to identify putative *Gl*Actin interactors and found them to localize to multiple regions within the cell, including the nuclei, plasma membrane, endomembrane system, marginal plate, flagella, and ventral disc. We also found 42 of previously validated *Gl*Actin-binding proteins (Table 2.1), as well as 21 proteins which had already been localized in live cells by the Dawson lab (UC Davis) as part of the *Giardia* genome database project (Table S2.3) [19]. 17 of the 46 proteins identified in our screen lack conserved domains or homologs in other organisms; thus, their protein sequences are not yet informative for deducing function. We also used I-TASSER to search for structural homologs [38], but none were identified. Of the proteins identified in our screen that contained conserved domains, six had ankyrin repeat domains, which have been found to serve as scaffolds for protein recognition and interaction and are present in actin regulators in other organisms [39,40]. We also found four alpha-giardins, annexin-like cytoskeletal proteins which have thus far only been found in *Giardia* [41]. The membrane-localized alpha-6 giardin may have a role in membrane organization, similar to the canonical annexin A-II, which binds F-actin and mediates its interactions with membranes [42,43].

Nuclear actin has been described in other eukaryotes, including mammals, plants, insects, and other protozoa [44]. Both monomeric and filamentous actin have functions within the nucleus. G-actin has a role in gene regulation through formation of the pre-initiation complex and in chromatin remodeling through its association with the BAF complex [44,45]. In contrast, F-actin has a role in intranuclear mobility, WNT-dependent transcription, and nuclear stability [16,44,46]. When immunostained, *Gl*Actin is visible in the nuclei; our lab previously demonstrated roles for *Gl*Actin in nuclear positioning and size [6,10,15]. Notably, most of these

functions depend on nuclear myosins, which are missing from the *Giardia* genome [3]; however, it is still possible that *GI*Actin fulfills these functions. Five of our putative F-actin interactors localized in distinct patterns to both nuclei; it will be interesting to determine which, if any, of these proteins play a role in nuclear shaping, size, or regulation of nuclear processes.

Actin also works not only within but immediately around the nucleus for protection in model organisms, as well as anchoring the nucleus and in nuclear positioning during mitosis, mediated by the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex [46,47]. While *Giardia* lacks the LINC complex [6], it is likely that actin is still involved in nuclear positioning, since *GI*Actin knockdowns results in mislocalized nuclei [6].

In model eukaryotes, the Arp2/3 complex drives actin polymerization to mediate clathrin-coated pit internalization, which is nucleated by the AP-2 complex [48]. Although *GI*Actin's role in membrane trafficking has been demonstrated, its possible involvement in clathrin-mediated endocytosis remains controversial [49,50]. *GI*Actin was not identified as an interactor of clathrin heavy chain in an earlier study [51], but other evidence points to their interaction [52]. Furthermore, depletion of *GI*Actin by morpholinos results in decreased uptake of Bodipy-LDL, which is mediated by clathrin [6,53]. The presence of clathrin light chain among our interactors points to some involvement. Due to the absence of most conserved endocytic proteins, the mechanism for clathrin-mediated endocytosis in *Giardia* is poorly understood. The clathrin heavy chain has been demonstrated to interact with components of the AP-2 adaptor complex, specifically alpha- and beta- adaptins, which localize to the plasma membrane [54]. The mu subunit of AP-2 has been localized to the hybrid endosome/lysosome peripheral vacuoles and plasma membrane [55]. It was therefore unexpected to see sigma adaptin, central to the structure of the AP-2 complex [56–58], localize to the nucleus in addition to the plasma membrane.

However, some of the other adaptins, notably alpha adaptin, have a function in nuclear translocation [59]; it is possible that both sigma adaptin and *GlActin* are involved in this process as well, particularly since sigma adaptin has both nuclear and plasma membrane localization.

Two other membrane-localized proteins, vacuolar protein sorting (VPS) 29 and 35, are subunits of the cargo recognition particle of the retromer complex. Retromer is involved in the endosome-to-Golgi retrograde transport pathway and mediates the recycling of membrane receptors in yeast and mammals [60]. Previous work in *Giardia*, which shows VPS29 localizing to the ER, conflicts with our results; however, the previous study was performed in fixed cells and fixation/permeabilization can sometimes disrupt membrane protein localization [61,62]. However, the same study localized VPS35 to the plasma membrane [63], so it is expected that at least a subset of VPS29 would also localize to the plasma membrane. While VPS26, the other subunit of the cargo recognition particle, did not fit our criteria for possible F-*GlActin* interactors, it did appear in our screen (Table S2.4). Since there is no Golgi in *Giardia* and the sorting nexins which constitute the other retromer subunit are also missing from the *Giardia* genome, the role of *Giardia's* cargo recognition particle is unknown. VPS35 has been shown to bind to a hydrolase receptor, but its function in doing so was not explored [63]. Furthermore, while the canonical VPS35 is known to coordinate with actin through interaction with FAM21 and the WASH complex [64], these are also absent in *Giardia*.

While the *Giardia* genome lacks moesin, ezrin, radixin, or any ERM domain proteins, *GlActin* does localize to the cellular cortex, so one or more of the putative interactors also localized here could fulfill the role of a cortical crosslinker. Furthermore, *GlActin*-depleted cells have defects in cell polarity and cell shape [6]. Another possible role for *GlActin* interactors localized to the plasma membrane is in phagocytosis, evidence for this process taking place in *Giardia* has

recently been published [65]; if further research shows *Giardia* to be capable of phagocytosis, *GI*Actin is likely to be involved [65,66], given its role in phagocytosis in other organisms.

A notable pattern in two cortical proteins, the alpha-giardin GL50803_14551 and the hypothetical protein GL50803_17255, was their reach into the ventrolateral flange (VLF). This structure is a membrane reservoir that skirts *Giardia* cells and contributes to attachment [34,35]. Short *GI*Actin filaments appear in the VLF, and another novel actin interactor, Flangin, has been implicated in its function [34]; it is likely that more of these interactors are important for maintenance and function of the VLF.

Many of our newly identified interactors localize to axonemes. Actin is a key component of the flagella inner dynein arm, although the actin within the inner arm is monomeric and complexed with a p28 homodimer [67]; additionally, actin appears to play a structural role in γ -TURC [68]. Immunostaining indicates *GI*Actin to be within the flagella and we have previously identified p28 as an interactor [22,69]. Actin's role in the flagella of other organisms is not merely structural; for instance, actin polymerization is required for flagellar biogenesis and intraflagellar transport in *Chlamydomonas reinhardtii* [70,71]. Morpholino knockdown of *GI*Actin results in flagella which are mispositioned or even missing, implicating *GI*Actin in flagellar positioning [6]. The two flagellar pore interactors we identified could also be involved in this process; future studies could investigate the role of these proteins through depletion to test if resulting flagella lack stability, are trapped within the cell body, or exit the cell at an incorrect location.

Despite the reduction of *Giardia*'s endomembrane system, it has a relatively conventional ER, and two of our proteins had ER-like localizations, consistent with *GI*Actin's previously established role in membrane trafficking. Two others, GL50803_12999 (hypothetical) and

GL50803_8044 (7 transmembrane domain) localize to an undescribed compartment. Since *Giardia* has unique organelles such as the Golgi-like encystation specific vesicles, it is likely that more compartments exist in *Giardia* than are currently recognized and these proteins may be involved in their function.

The marginal plate, a crescent-shaped structure at the anterior of the cell, has not yet been functionally studied, limiting our ability to infer the function of the proteins located there. While it has been suggested to be involved in attachment, this role is speculative [72]. Since immunofluorescence assays show enrichment of *GI*Actin at this site, it is possible that the meshwork of filaments seen in electron micrographs [72] are composed of *GI*Actin or that *GI*Actin acts upon this structure. Intriguingly, after depletion, *GI*Actin localization persists at the marginal plate, suggesting that the associated *GI*Actin is highly stabilized [15].

Of all the structures to which these putative *GI*Actin interactors localize, the ventral disc is perhaps the most surprising. One previous study localized *GI*Actin to the ventral disc [73]; however, this study used a heterologous antibody, raised against chicken gizzard actin, for immunostaining. [73,74]. While the disc itself is composed of microtubules, their polymerization dynamics are not responsible for flexation and/or attachment; it is possible that *GI*Actin, as a force-generating protein, plays a role in this process. *Giardia*'s ability to attach, and thus its health, depends on the disc, and as this structure is unique to *Giardia*, many of the proteins that localize there have no known relationship to proteins in other organisms. It is noteworthy that the different disc proteins had different localizations, indicating that the different regions of the disc may have different relationships to *GI*Actin. Perhaps most notable are GL50803_16844 and GL50803_113644, as they are highly enriched in the margin near the overlap zone and/or the ventral groove; these regions are hypothesized to regulate fluid flow beneath the ventral disc

[75]. While *GI*Actin does not appear concentrated in the ventral disc in immunofluorescence images, this could be due to steric hindrance between the antibody and proteins on the ventral disc, similar to the faint tubulin immunostaining seen in the disc compared to the amount of signal observed when using fluorescently-tagged tubulin [37]. Another possibility is that F-actin is localized to the disc, but it is obscured by the pool of monomeric *GI*Actin in our immunostaining. Defects in one of our disc-localized interactors, gamma-giardin, results in misshapen discs, but normal adherence, suggesting the role of gamma-giardin to be structural rather than actively mechanical [76]. Notably, over half of our interactors with previously reported localizations are at the ventral disc (Table S2.3) [19,77].

Here we have characterized a number of putative *GI*Actin interactors, with a diversity of localizations and possible applications. Study of *GI*Actin is challenging as we still lack an actin reporter for live imaging in *Giardia*, and markers that generally recognize actin, such as LifeAct, F-tractin, and Utrophin, in other eukaryotes do not label filamentous structures in *Giardia*. The actin-binding domains of the proteins identified in our study could be mapped and potentially used as *in vivo* *GI*Actin markers. Of particular interest are the proteins which localize to the ventral disc, the ventrolateral flange, and the marginal plate. If, as hypothesized, the plate is important for attachment, our work has potentially uncovered a connection between *GI*Actin and *Giardia*'s mechanisms for attachment, which is required for maintaining infection. Thus, there are ample opportunities for drug development in further study of these proteins, particularly since few of them have homologues in humans thereby reducing the likelihood of unwanted effects.

Materials and Methods

Parasite Strain and Growth Conditions

G. lamblia strain WB clone 6 (ATCC 50803; American Type Culture Collection) was cultured under standard conditions [78].

Lysis and Crosslinking

3 L of wild type *Giardia* trophozoite culture was grown to confluence in 1 L bottles as described previously [10]. Cells were iced for 2 hours to induce detachment, then centrifugation for 30 minutes at 1500xg at 4° C, then washed twice with 1XHBS (HEPES buffered saline) plus 1xHALT protease inhibitor cocktail (Thermo Scientific Catalog number 78430), followed by resuspension and centrifugation for 15 minutes at 1500xg at 4°C. Each culture was then resuspended in 1.6 mL of buffer. Two were resuspended in F-buffer (20 mM HEPES, 0.2 mM CaCl₂, 80 mM KCl, 10 mM imidazole, 1 mM MgCl₂, 1 mM EGTA, 5% Glycerol, 10 mM ATP, 1X HALT protease inhibitors, pH 7.2) while one was resuspended in G-Buffer (20 mM HEPES, 0.2 mM CaCl₂, 0.2 mM ATP, 1X HALT protease inhibitors, pH 7.2). One of the F-buffer cultures was then treated with crosslinker DSP (dithiobis(succinimidyl propionate)) (Thermo Scientific Catalog number 22585) to a final concentration of 1 mM and incubated for 30 minutes at room temperature, quenched with Tris-HCl pH 7.5 to a final concentration of 20mM and incubated at 15 minutes, then lysed in the manner described below. The other F-buffer culture and the G-buffer culture were lysed with sonication, pulse for 25 seconds each at 20% power with 1-minute rest between each sonication, repeated a total of 4 times minimum. Sonicated cells were allowed to rest on ice for 30 minutes, then cleared with a 10,000xg spin for 10 minutes at 4°C. The supernatant of these two cultures was then treated with DSP crosslinker to a final

concentration of 1 mM and incubated for 30 minutes at room temperature, quenched with Tris-HCl pH 7.5 to a final concentration of 20mM and incubated at 15 minutes. This was repeated for a total of three independent replicates per condition.

Purification and Sample Preparation for Mass Spectrometry

Lysates were incubated overnight with an anti-*Gl*Actin antibody [6] at 4°C, then incubated with 500 µL Pierce™ Protein A agarose (Catalog number 20333) bead slurry and incubated with end-to-end mixing for 2 hours at room temperature. A total of four washes were performed by addition of 0.5 mL of wash buffer (G- or F-Buffer with an additional 150 mM NaCl), vortexed, and centrifuged for 2-3 minutes at 2500xg. For elution, 250 µL of 0.2 M Glycine pH 2.5 was added to the beads and incubated for 5 minutes, centrifuged for 3 minutes at 2500xg, and the supernatant was collected. This step was then repeated and the two eluate fractions were combined. The pH was then neutralized through addition of 50 µL 1 M Tris-HCl pH 7.5.

For each condition, 5X sample buffer (0.225 M Tris-HCl, pH 6.8, 50% glycerol, 5% SDS, 0.05% bromophenol blue, 0.25 M DTT) was added to a final concentration of 1X subsequent to running on a 10% SDS-PAGE gel and stained with SYPRO Ruby (Pierce). Bands corresponding to the heavy and light antibody chains were excised from the gel and discarded; the remainder of the gels were divided into rectangles of approximately equal size. These rectangles were cut into squares of approximately 1-2 mm, washed with 200 µL water, then incubated with 200 µL of 50% acetonitrile and 25 mM ammonium bicarbonate for 5 minutes. After addition of another 200 µL acetonitrile and further incubation for 1 minute, the gel was dried with a speed vacuum, followed by another addition of 50 µL of 25 mM ammonium bicarbonate and 10 mM TCEP.

Samples were then incubated at 60°C for 1 hour, after which the supernatant was removed and replaced with 50 µL of 25 mM ammonium bicarbonate and 10mM iodacetamide, then incubated for 20 minutes in the dark. Another wash was performed with 400 µL water and the 5-minute incubation with 200 µl 50% acetonitrile, 25 mM ammonium bicarbonate and following steps were repeated. Protein samples were then digested with 20 µL of 0.01 µg/µL Promega Trypsin in 25 mM ammonium bicarbonate. More ammonium bicarbonate was added to cover, followed by overnight digestion. Supernatant was then removed for LC/MS-MS analysis. Protein was further extracted from the gels by addition of 50 µL of acetonitrile, vortexing, and removing and saving supernatant. One last extraction was performed by addition of 50 µL 60% acetonitrile, 0.1% formic acid, vortexing for 5 minutes, and then removing the supernatant. The supernatants were then combined, dried, and resuspended in solvent A, then analyzed by LC/MS-MS as described below.

Liquid Chromatography and Mass Spectrometry

Liquid chromatography – mass spectrometry was performed on a Velos Pro (Thermo) with an EasyLC 1000 HPLC and autosampler (Thermo). Samples were solubilized in loading buffer (0.1% trifluoroacetic acid and 2% acetonitrile in water), and 6 µl was injected via the autosampler onto a 150-µm Kasil fritted trap packed with Reprosil-Pur C18-AQ (3-µm bead diameter, Dr. Maisch) to a bed length of 2 cm at a flow rate of 2 µl/min. After loading and desalting using a total volume of 8 µl of loading buffer, the trap was brought on-line with a pulled fused-silica capillary tip (75-µm i.d.) packed to a length of 25 cm with the same Dr. Maisch beads. Peptides were eluted off the column using a gradient of 2-35% acetonitrile in 0.1% formic acid over 90 minutes, followed by 35-60% acetonitrile over 5 minutes at a flow rate

of 250 nl/min. The mass spectrometer was operated using electrospray ionization (2 kV) with the heated transfer tube at 200 C using data dependent acquisition (DDA), whereby a mass spectrum (m/z 400-1600, normal scan rate) was acquired with up to 15 MS/MS spectra (rapid scan rate) of the most intense precursors found in the MS1 scan. Database searches were performed using Comet [79] searched against the protein sequence database GiardiaDB

3.1_GintestinalisAssemblageA_AnnotatedProteins.fasta to which was appended sequences of common contaminants (e.g., human keratins). The peptide mass tolerance was 3 Da, and the fragment ions were sorted into 1 Da bins (roughly corresponding to a fragment ion mass tolerance of ± 0.5 Da). Semi-tryptic cleavages and up to two missed tryptic cleavage sites were allowed. Oxidized methionine and acetylation of the protein N-termini were allowed as variable modifications. Carbamidomethylated cysteine was a fixed modification. False discovery rates (1%) were determined using PeptideProphet [80], and protein inferences were made using ProteinProphet [81]. Spectral count analysis was performed using Abacus [82].

Bioinformatics

Delta-BLAST, Protein BLAST [85], and Pfam [86] searches were used to identify homologues and conserved domains for each putative interactor. I-TASSER was used to search for structure-based function prediction [38].

Data Availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange [83] Consortium via the PRIDE partner repository with the dataset identifier PXD026067 [84].

Vector Construction

All constructs in this study were C-terminal fusions with the exception of the N-terminally tagged GL50803_33989 and GL50803_7323, using Gibson reactions with linearized vectors and PCR products [7,87]. For primer sequences and workflow, see

Table S2.5.

Immunofluorescence Microscopy

Fixation and imaging were performed as described in previous work [88]. FIJI/ImageJ were used for image analysis, including co-localization analysis by Just Another Colocalization Plugin (JACoP) [89]. Three images were analyzed for each protein.

Live Cell Imaging

Cells were chilled with ice for 15 minutes to detach from the culture tube and then placed into an Attofluor cell chamber (Molecular Probes) and incubated in a GasPak EZ anaerobic pouch (BD) or a Tri-gas incubator (Panasonic) set to 2.5% O₂, 5% CO₂ for 90 minutes at 37° C. Cells were then washed four times with HEPES-buffered saline (137mM NaCl, 5mM KCl, 0.91mM Na₂HPO₄-heptahydrate, 5.5mM Glucose, 20mM HEPES, and pH7), overlaid with a mixture of 0.7% ultra-low gelling agarose (Sigma A2576) melted in HEPES-buffered saline, cooled for 10 minutes at room temperature to solidify, then imaged. Live cell imaging was performed on a DeltaVision Elite microscope (GE) equipped with DIC optics, using a 100X 1.4 NA or 60X 1.42 NA objective, and a sCMOS 5.4 PCIe air-cooled camera (PCO-TECH).

Co-immunoprecipitation

500 mL of *Giardia* cell cultures were grown for 3 days, then iced for 2 hours to detach and spun for 1500xg at 4 °C. Cells were then washed twice in HBS with 2XHALT protease inhibitors and

10 μ M chymostatin, 1 μ M leupeptin, and 1 μ M E64. Each pellet was resuspended to a final volume of 1.2 mL and 100 mM DSP in DMSO was added to a final concentration of 1 mM and incubated at room temperature for 30 minutes. The reaction was quenched for 15 minutes with an addition of Tris-HCl pH 7.4 final concentration 20 mM. Cells were then pelleted by spinning for 7 minutes at 700xg and resuspended in 350 μ L lysis buffer (80 mM KCl, 10 mM imidazole, 1 mM MgCl₂, 1 mM EGTA, 5% Glycerol, 20 mM HEPES, 0.2 mM, CaCl₂, 10 mM ATP, 0.1% Triton X-100, 500 mM NaCl, pH 7.2). Cells were then lysed by sonication and cleared as described above. A volume of 17.5 μ L of equilibrated EZview Red Anti-HA Affinity gel (Sigma) was added to each tube of lysate, then incubated at 4°C with end-over-end mixing for 1 hour. Beads were then spun at 8,200xg for 30 seconds and the supernatant was discarded, followed by a total of three washes with 750 μ L wash buffer (80 mM KCl, 10 mM imidazole, 1 mM MgCl₂, 1 mM EGTA, 5% Glycerol, 20 mM HEPES, 0.2 mM CaCl₂, 10 mM ATP, 0.5% Tween, 500 mM NaCl, pH 7.2). Each wash consisted of end-over-end rotation for 5 minutes followed by a 30 second spin at 8,200xg. Protein was then incubated with 50 μ L of 8 M Urea at RT for 20 minutes to elute, followed by addition of 5X sample buffer (QIAGEN) to a final concentration of 1X, boiled for 5 mins at 98° C, and run on 12% SDS-PAGE gel, followed by Western blotting protocol described previously [7]. Representative images from three replicates are shown for each experiment.

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2.1 CHAPTER 2 TABLES AND FIGURES

Table 2.1. Proteins identified as putative F-Actin interactors

ORF Number	Identity	Localization
Plasma Membrane/Cortical Localization		
GL50803_8560	Hypothetical	Plasma membrane
GL50803_103855	VPS29	Plasma membrane
GL50803_23833	VPS35	Plasma membrane
GL50803_4259	Clathrin Light Chain	Plasma membrane
GL50803_8044	7-transmembrane domains	Plasma membrane and unidentified compartment
GL50803_15591	Coiled coil protein	Plasma membrane
GL50803_14551	Alpha-6 giardin	Ventrolateral flange and plasma membrane
GL50803_17255	Hypothetical	Plasma membrane, flange, basal bodies
GL50803_5810	Hypothetical	Plasma membrane
GL50803_11684	Leucine Rich Repeat Protein	Plasma membrane
Internal Membrane Localization		
GL50803_9861	Hypothetical	Diffuse internal membrane
GL50803_13930	Arf3	Puncta
GL50803_22855	Hypothetical	Vesicles
GL50803_12999	Hypothetical	Puncta and unidentified compartment
Marginal Plate Localization		
GL50803_17153	Alpha-11 giardin	Marginal plate and axonemal-associated structures
GL50803_115478	Ankyrin repeat, takusan, SMC	Marginal plate and flagellar exit sites
GL50803_8854	Hypothetical	Marginal plate and flagellar exit sites
GL50803_4383	Ankyrin repeat	Marginal plate and axonemal-associated structures
GL50803_5800	Hypothetical	Marginal plate and puncta
GL50803_9030	Ankyrin repeat	Marginal plate and cytoplasmic puncta
GL50803_16522	Hypothetical	Cytoplasmic, marginal disc, nuclei

Nuclear Localization		
GL50803_101212	Hypothetical	Perinuclear region
GL50803_33989	Hypothetical	Puncta and filaments within nucleus
GL50903_14299	Hypothetical	Diffuse nuclear localization with puncta
GL50803_5328	Sigma adaptin (AP2 complex)	Diffuse nuclear localization and plasma membrane
GL50803_102813	Ankyrin repeat	Nuclear, marginal plate, cytoplasmic
GL50803_14213	Hypothetical	Diffuse nuclear localization with puncta
Flagellar Localization		
GL50803_9848	Dynein light chain	Internal portions of flagella, basal bodies
GL50803_33685	Armadillo repeat	Flagella
GL50803_15097	Alpha-14 giardin	Flagella
GL50803_13774	Hypothetical	Flagellar exit sites
GL50803_4463	Dynein light chain	Flagella
GL50803_15124	Dynein light chain	Flagella
Disc localization		
GL50803_4239	Hypothetical	Ventral disc, cytoplasmic
GL50803_24451	FGF	Flagella, basal bodies, lateral crest
GL50803_8726	Hypothetical	Ventral disc, midbody
GL50803_27925	Ankyrin repeat	Ventral disc, except ventral groove
GL50803_113622	Ankyrin repeat	Ventral groove
GL50803_16844	Hypothetical	Ventral disc: ventral groove and overlap zone
GL50803_17230	Gamma-giardin	Ventral disc
GL50803_11354	Hypothetical	Flagella, lateral crest
Non-specific localization		
GL50803_10038	Alpha-18 giardin	Diffuse non-specific
GL50803_10429	Wos2 protein	Diffuse non-specific
GL50803_7323	Hypothetical	Diffuse non-specific
GL50803_6242	Translationally controlled tumor protein-like	Diffuse non-specific
GL50803_21423	Beta-adaptin	N/A

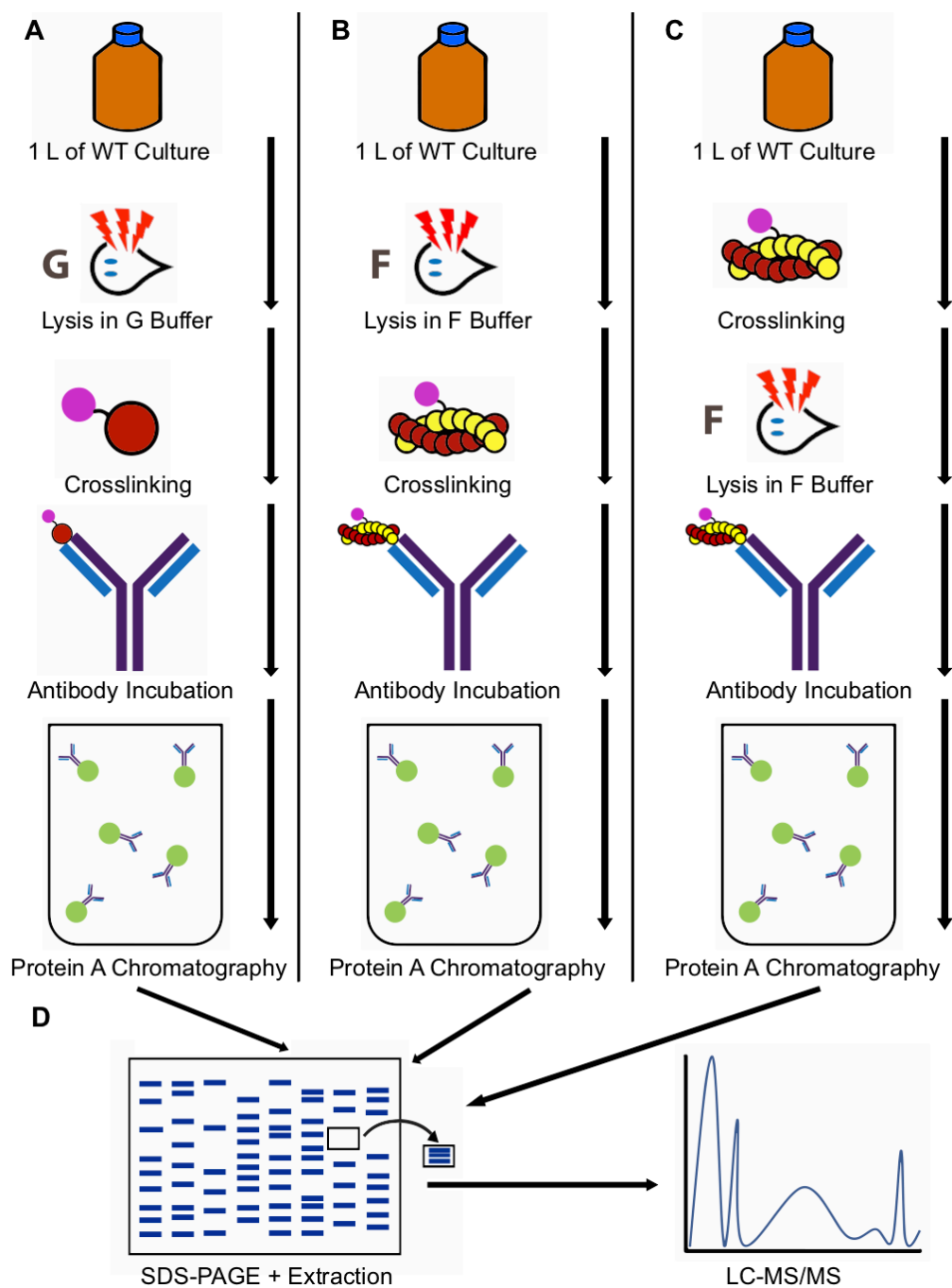


Figure 2.1. Schematic of methods.

1 L of cells was lysed under one of the following conditions: (A) in G-buffer followed by crosslinking, (B) F-buffer followed by crosslinking, (C) or treated with crosslinker, then lysed in F-buffer. Each lysate was then incubated with an anti-GlActin antibody followed by Protein A chromatography. (D) The eluate was run on an SDS-PAGE gel, excised, and identified by LC-MS/MS.

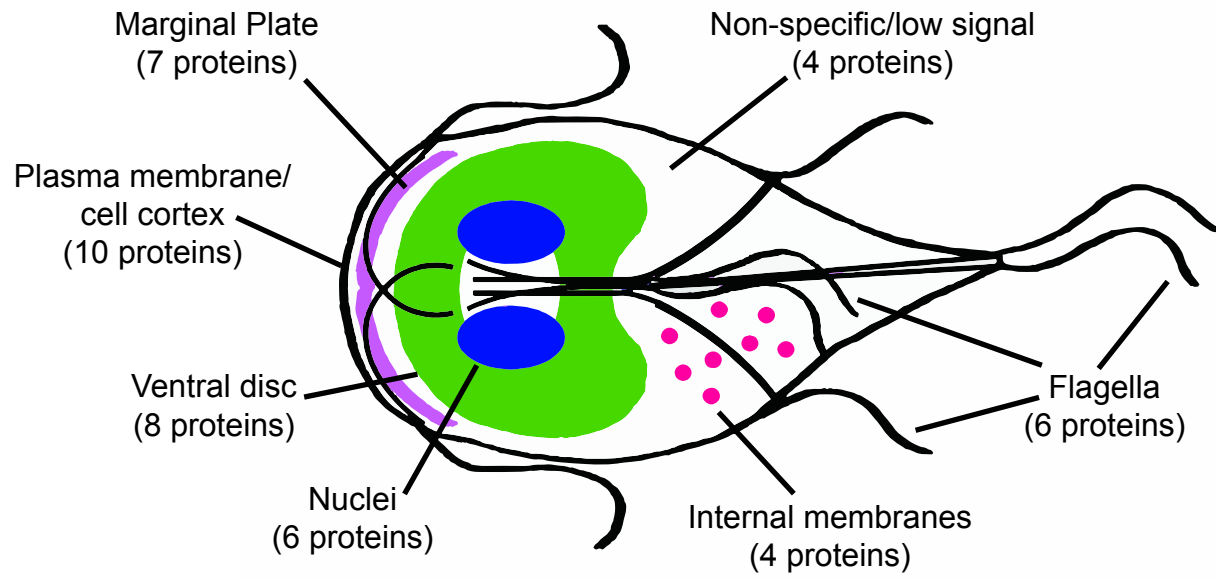


Figure 2.2. Localization of 45 *GlActin* Filament Interactors.

Diagram of *Giardia* cell, showing the nuclei (blue), ventral disc (green), marginal plate (purple), flagella and axonemes (black), internal membranes, (pink), plasma membrane (black), and non-specific/low signal. The number of interactors localizing to each structure is reported.

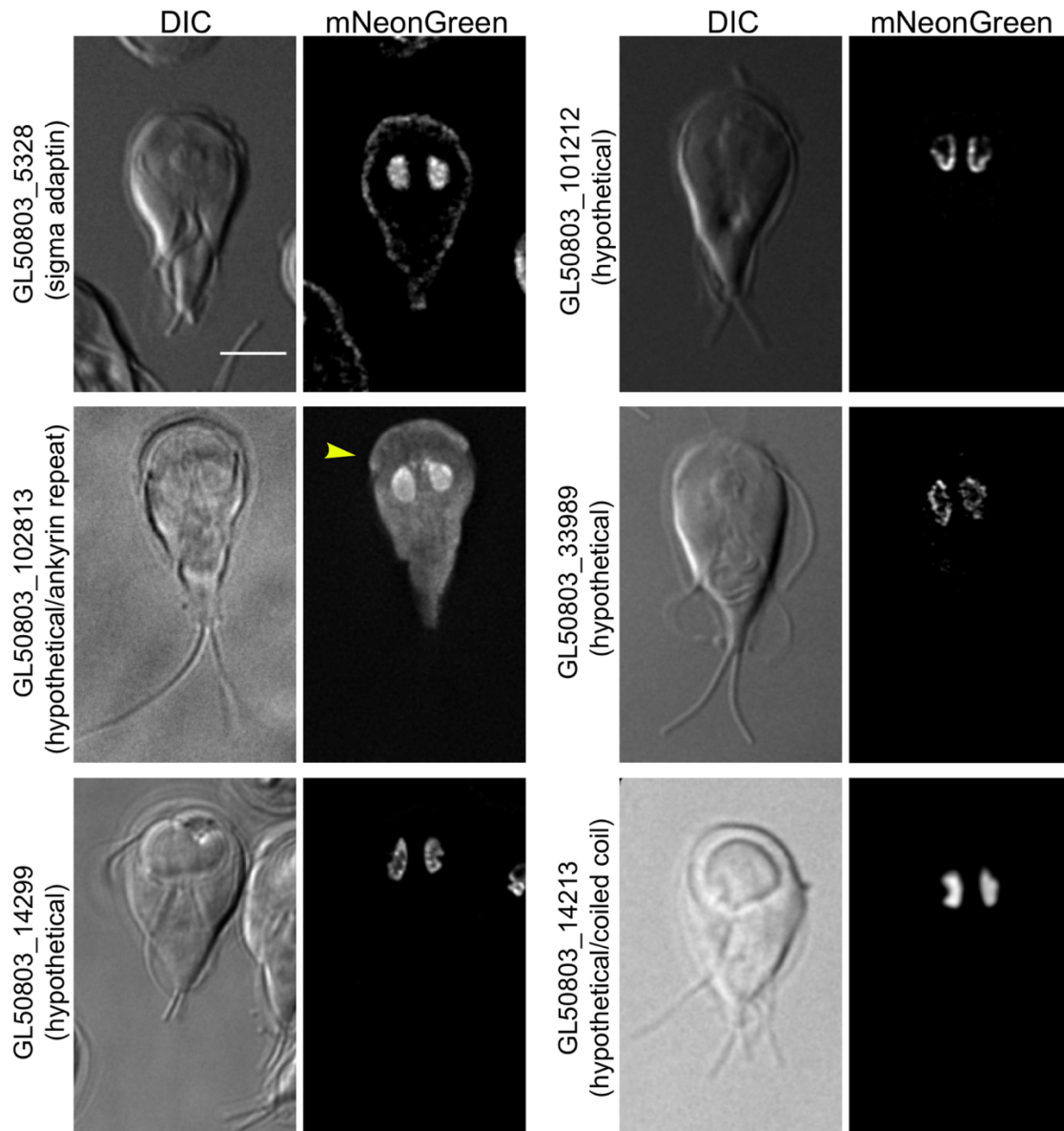


Figure 2.3. Putative *GI*Actin interactors with nuclear enrichment

Six proteins identified were enriched in *Giardia*'s nuclei, based on the localization of mNeonGreen fusions. GL50803_5328 also localized to the cell cortex. GL50803_102813 was also enriched in the marginal plate (yellow arrow) and cytoplasm, with some puncta. GL50803_GL14299 and GL50803_33989 appeared closer to the nuclear envelope, while GL50803_101212 localized to the perinuclear region. GL50803_14213 was evenly distributed within the nuclei. Scale bar denotes 5 μm .

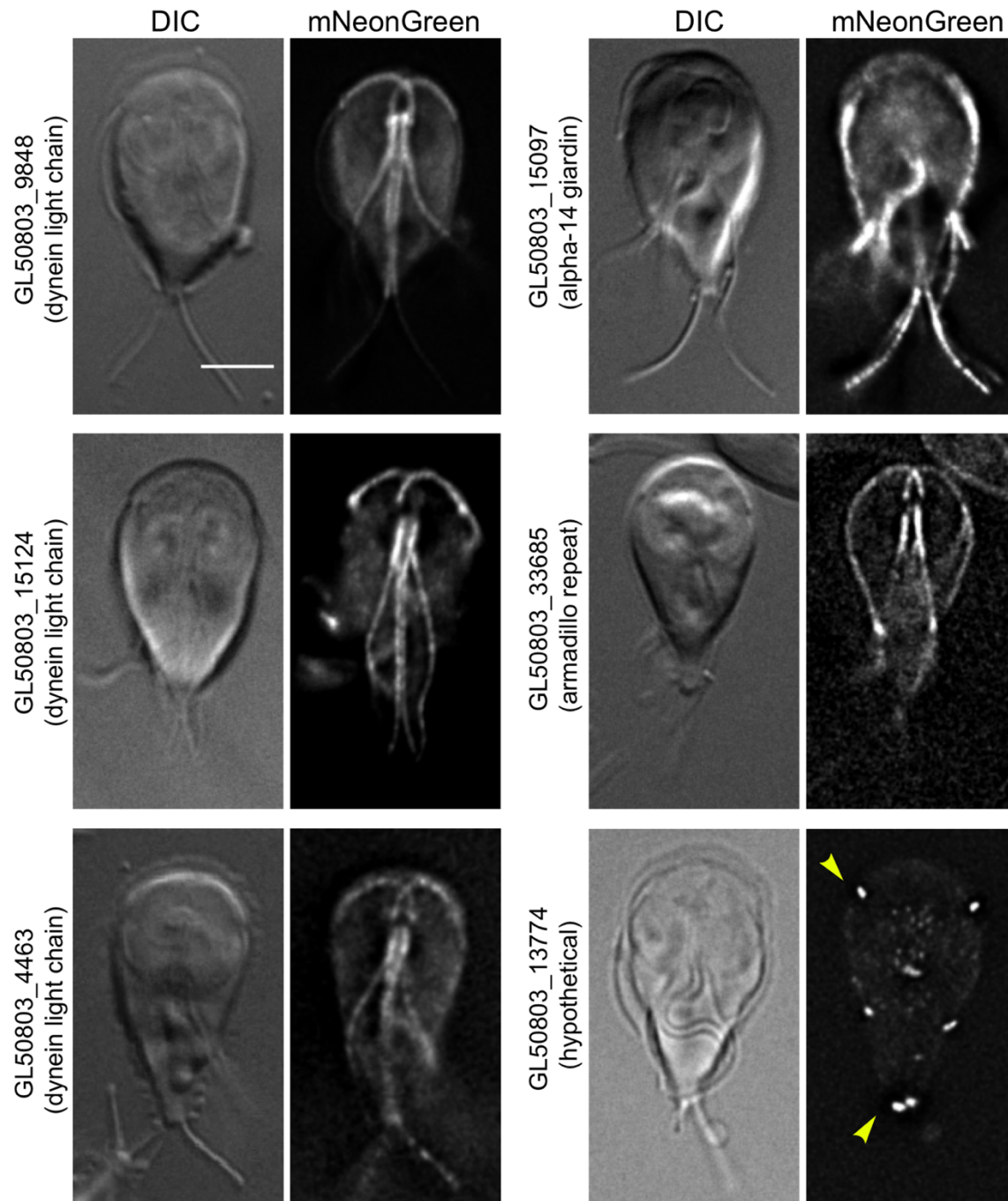


Figure 2.4. Putative *GI*Actin interactors with flagella localization

Six *GI*Actin interactors localized to flagella or flagella-related structures based on the localization of mNeonGreen fusions. GL50803_15124 had a general flagellar localization. GL50803_4463 was enriched in the central axonemes, while GL50803_9848 was enriched in the basal bodies. GL50803_15097 was enriched in posterolateral, ventral, and caudal flagella. GL50803_33685 also appeared slightly in the plasma membrane. GL50803_13774 was only present in the flagellar pores (yellow arrows). Scale bar denotes 5 μ m.

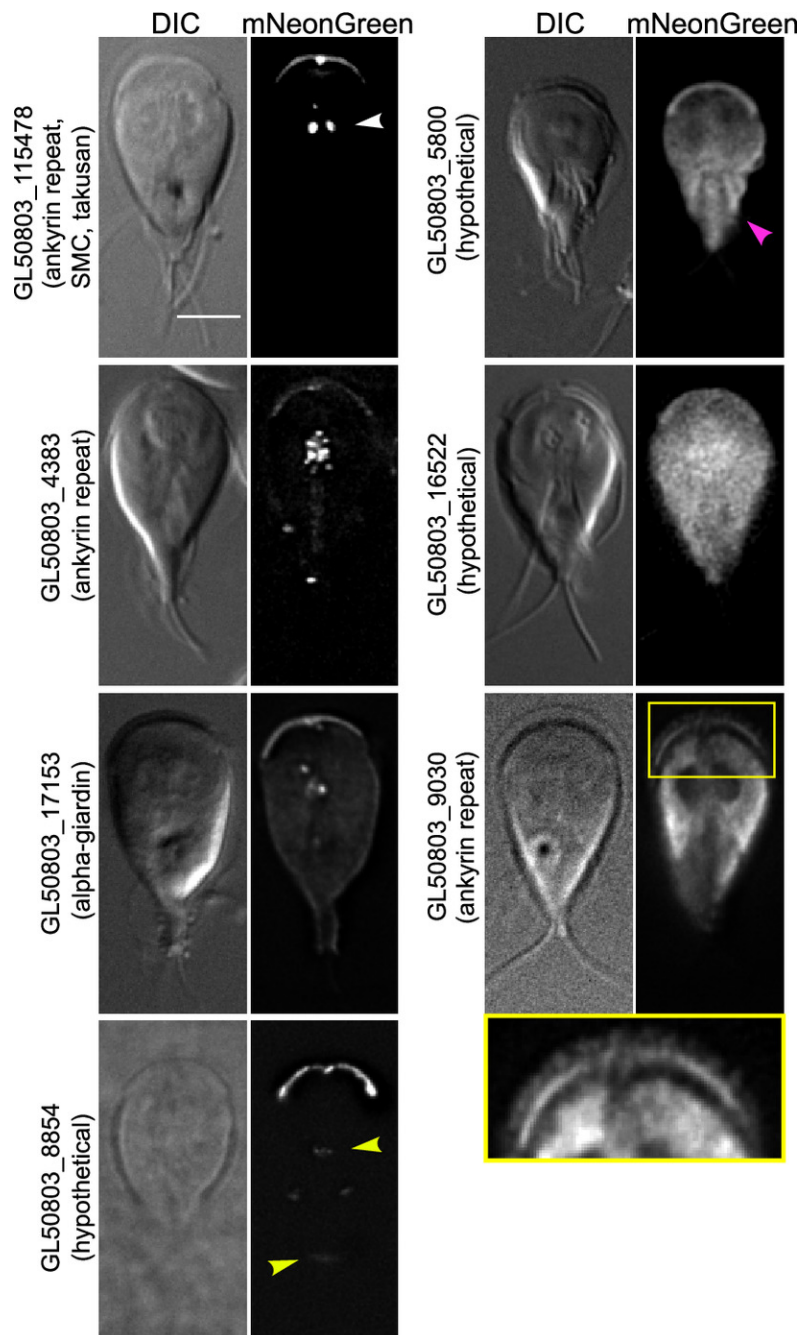


Figure 2.5. Putative *G/Actin* interactors with marginal plate localization

Seven *G/Actin* interactors localized to the marginal plate based on the localization of mNeonGreen fusions. GL50803_115478, GL50803_17153, and GL50803_4383 also appeared in axoneme-associated structures (white arrow). GL50803_8854 was enriched in the flagellar pores (yellow arrows). GL50803_5800 also appeared in the axonemes (magenta arrow) and, similar to GL50803_16522, was only slightly enriched in the plate. GL50803_9030 was additionally observed in linear arrays that may be filaments at the anterior ventrolateral flange and also localized throughout the cytoplasm with enrichment in the lateral shields, but was excluded from the nuclei. Scale bar denotes 5 μ m.

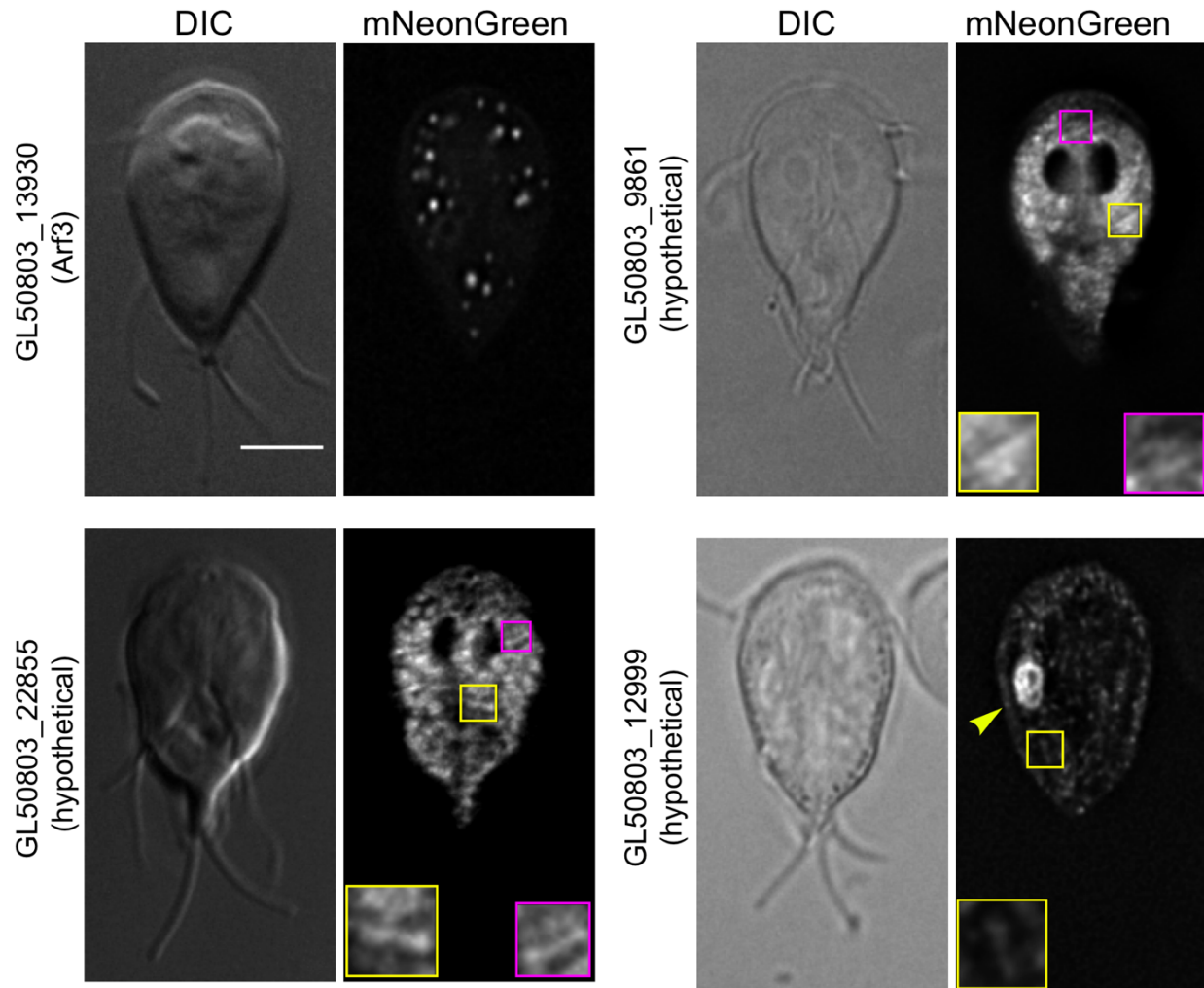


Figure 2.6. Putative GActin interactors in the endomembrane system

Four GActin interactors localized to internal structures likely to be the endomembrane system. GL50803_13930 was concentrated in a few stable puncta, while GL50803_22855 and GL50803_9861 were widely distributed throughout the cytoplasm. GL50803_12999 localized to cytoplasmic puncta and a large, oblong organelle (yellow arrow). Except for Arf3, these proteins are sometimes seen in linear arrays (shown in insets) that could also indicate a connection to actin filaments. Scale bar denotes 5 μm .

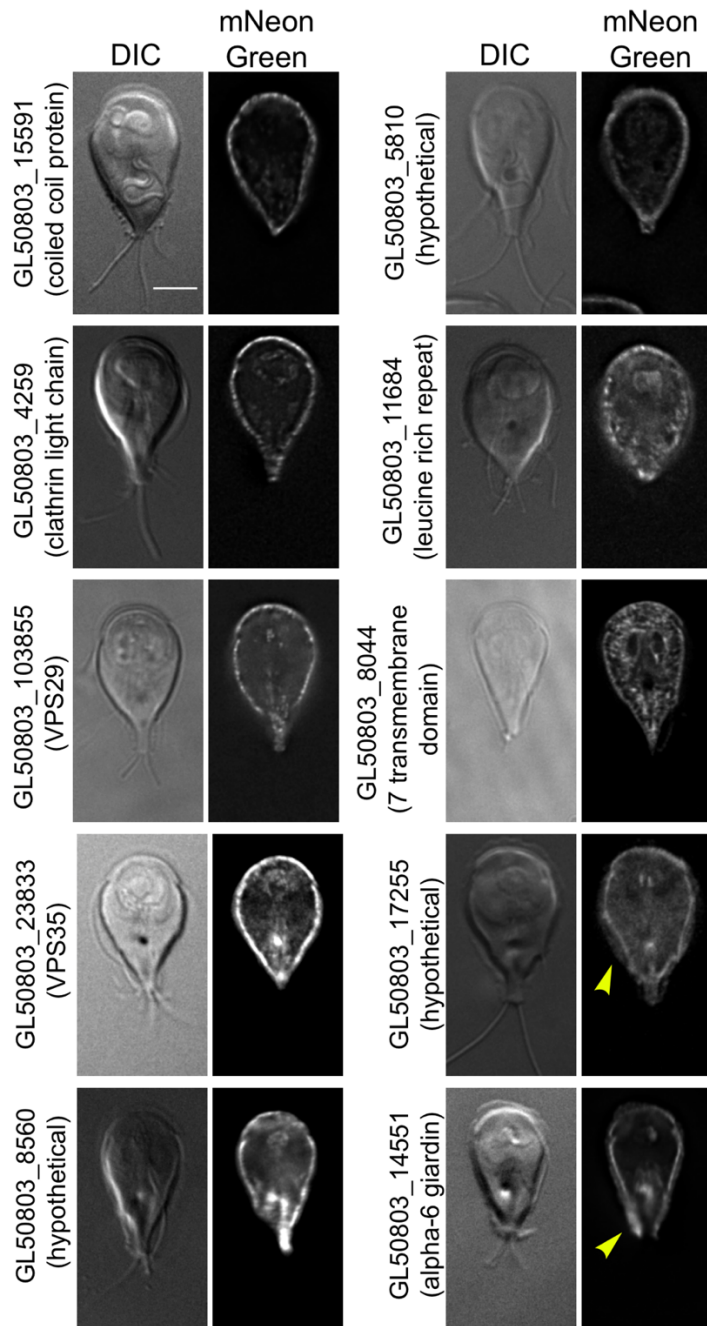


Figure 2.7. Putative *G/Actin* interactors in the cortex and plasma membrane

Ten *G/Actin* interactors localized to the cell cortex based on the localization of mNeonGreen fusions. Clathrin light chain GL50803_4259 is expected at the cell cortex. GL50803_5810 also had a slight localization to the nuclear envelope and GL50803_103855, GL50803_23833, and GL50803_8560 had a slightly punctate distribution within the plasma membrane. GL50803_15591 and GL50803_11684 were even more punctate, with individual puncta clearly visible. GL50803_8044 localized to the ER. Both GL50803_14551 and GL50803_17255 extended into the ventrolateral flange (yellow arrows); the latter was also enriched in the basal bodies. Scale bar denotes 5 μm .

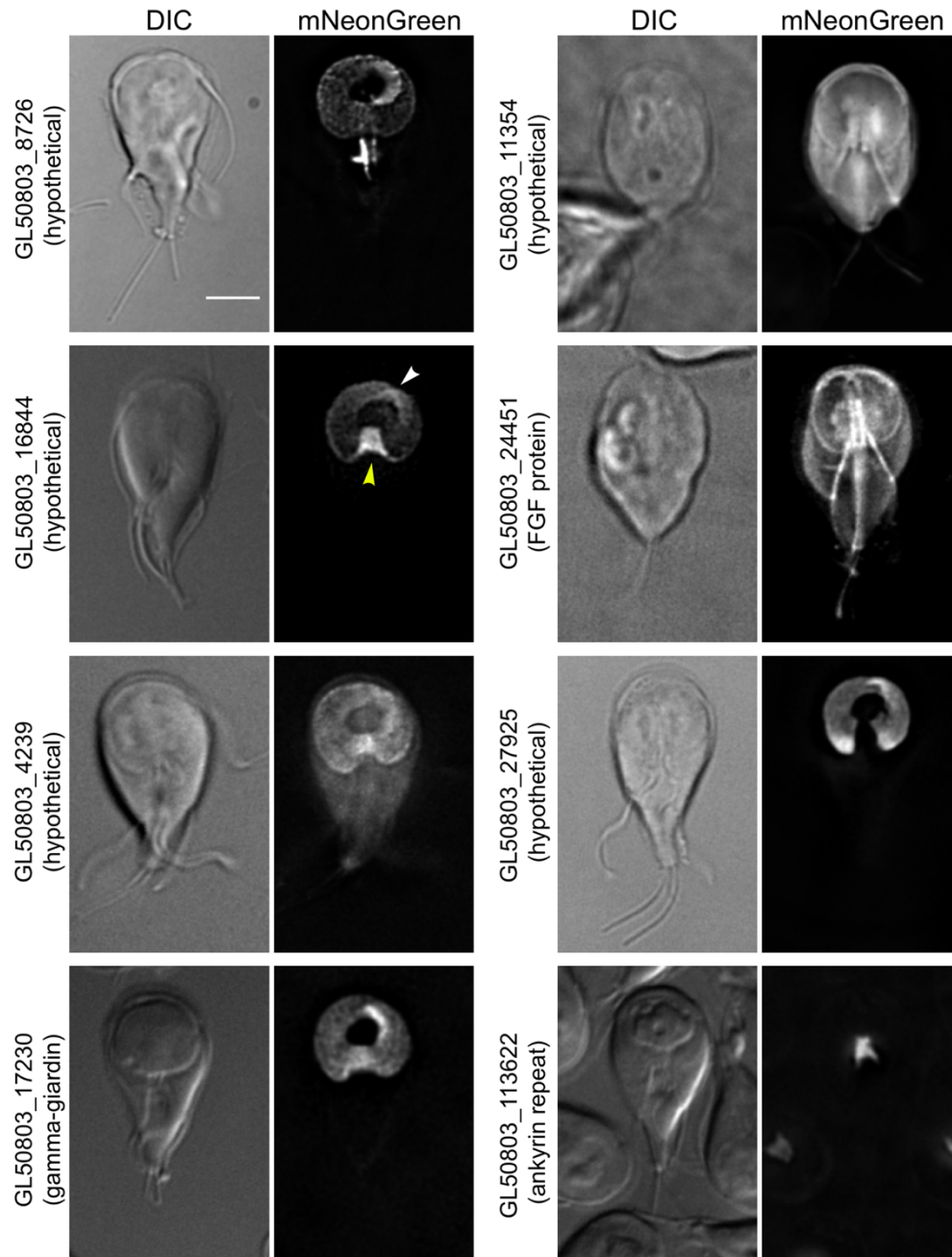


Figure 2.8. Putative *G/Actin* Interactors with ventral disc localization

Eight proteins identified localized to the ventral disc based on mNeonGreen fusions.

GL50803_8726 was more prominent on the disc margin and the overlap zone and appeared on the median body. GL50803_16844 was enriched in the ventral groove (yellow arrow) and the overlap zone (white arrow); GL50803_17230 was similarly enriched in the overlap zone. GL50803_4239 had a slightly brighter signal in the ventral groove, the only place where GL50803_113622 localized. GL50803_11354 and GL50803_24451 faintly outlined both the ventral disc and the flagella. GL50803_27925 is excluded from the ventral groove. Scale bar denotes 5 μ m.

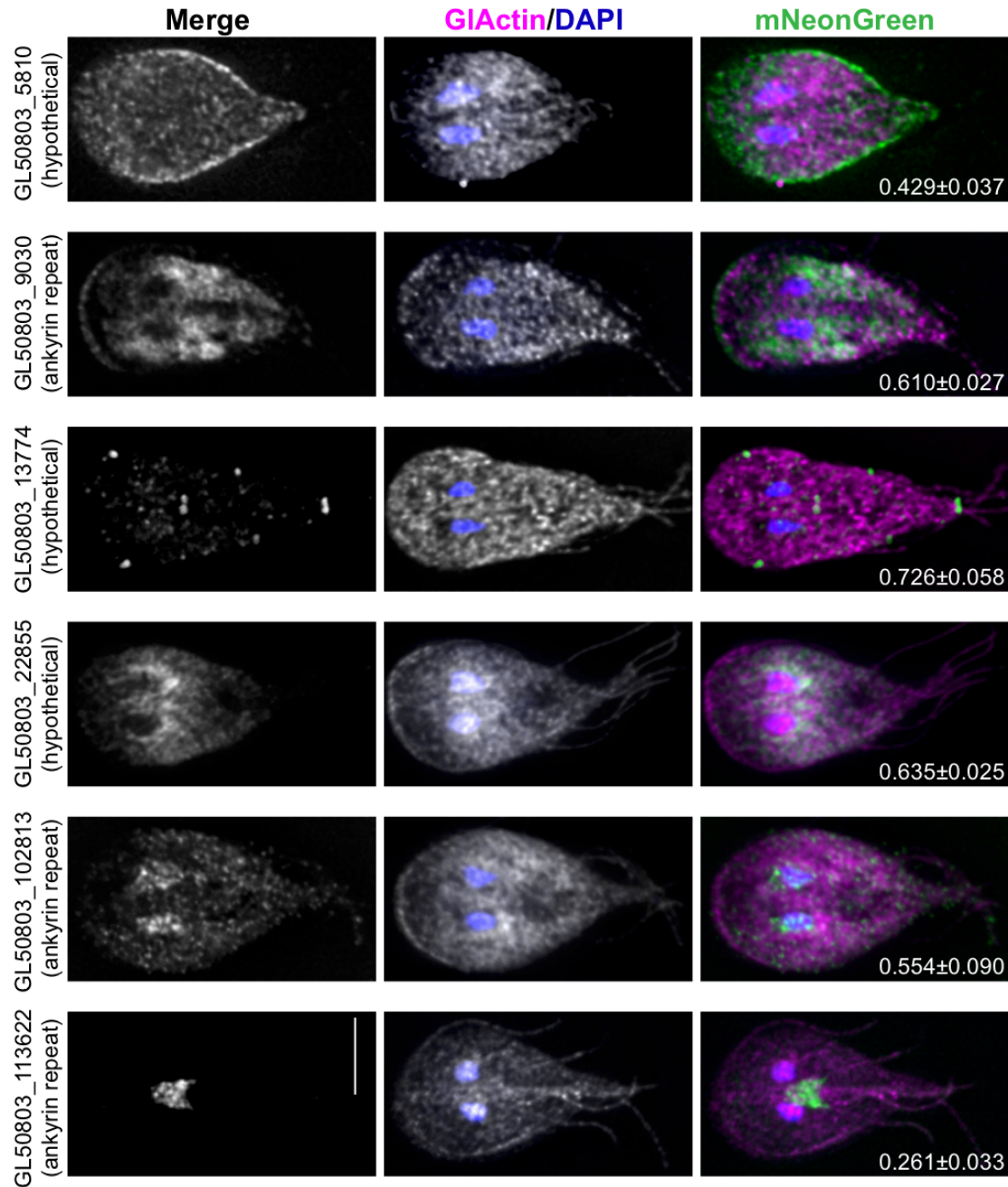


Figure 2.9. Immunostained *GI*Actin is localized throughout the cell

One protein from each category of localization was co-localized with *GI*Actin in fixed *Giardia* trophozoites. Nuclei are stained with DAPI (blue), *GI*Actin is magenta, and each mNeonGreen-tagged interactor is in green. Average projections are shown. Scale bar denotes 5 μ m. Pearson's correlation coefficients using Costes randomization were calculated for three images of each protein, shown as mean $\pm$ SD on each merge. The Costes P-value was 1 for all candidates.

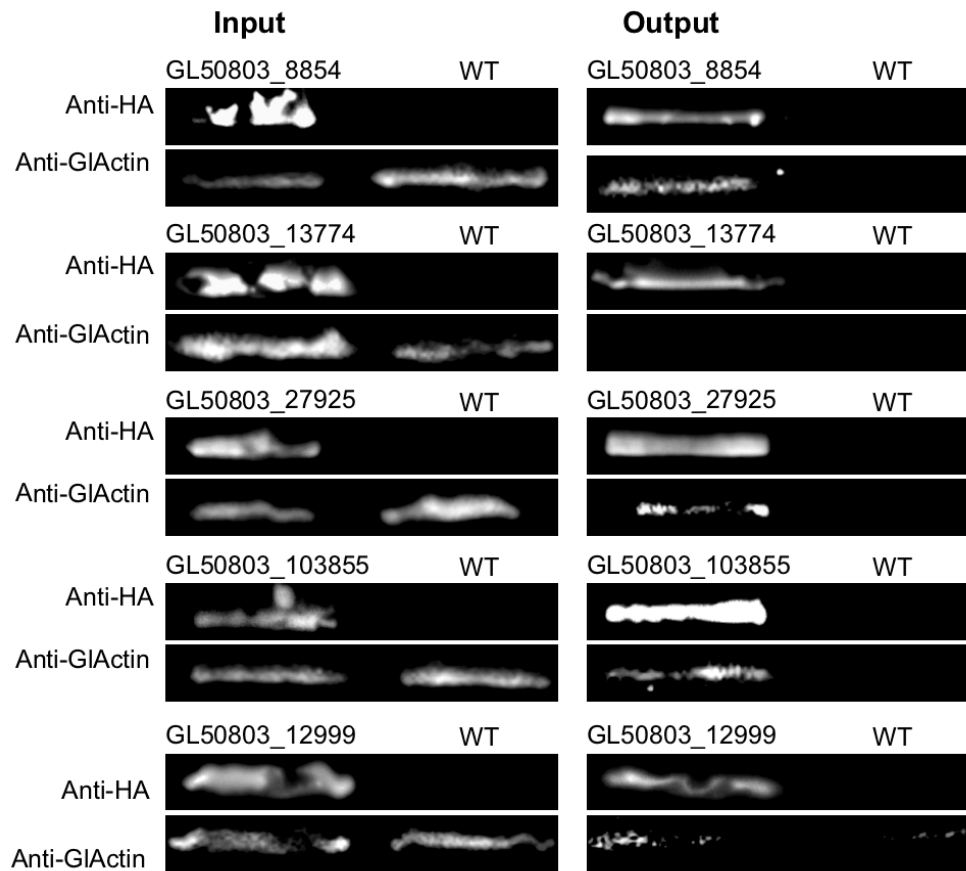


Figure 2.10. Reciprocal co-immunoprecipitation

One protein from each category of localization was tagged with 3xHA and immunoprecipitated, followed by anti-*GlActin* Western blotting. Three of the six proteins tagged definitively complexed with *GlActin*, including GL50803_8854 (marginal plate), GL50803_103855 (plasma membrane), and GL50803_27925 (ventral disc). GL50803_33989 (nuclear) was too low expression to be visible on western blot, and is thus not shown.

Table S2.2. Previously validated *GL*Actin interactors identified in this screen

ORF Number	Name	Identity	Conditions and number of replicates			
			F-Buffer	G-Buffer	DSP Pre-treatment	Dominant Buffer Condition
GL50803_111950	Axonemal Dynein Heavy chain	Axonemal	0	1	0	G
GL50803_101138	Axonemal Dynein Heavy chain	Axonemal	0	1	0	G
GL50803_40496	Axonemal Dynein Heavy chain	Axonemal	1	2	2	G and DSP pre-treatment
GL50803_13273	Axonemal Dynein Heavy chain	Axonemal	3	3	3	G, F, and DSP pre-treatment
GL50803_42285	Ciliary dynein heavy chain 11	Axonemal	0	1	0	G
GL50803_6744	Centrin	Axonemal	0	2	0	G
GL50803_37985	Dynein heavy chain	Axonemal	0	1	0	G
GL50803_137716	Axoneme-associated protein GASP-180	Axonemal	3	3	3	G and DSP pre-treatment
GL50803_14242	Dynein heavy chain-cytoplasmic	Axonemal	0	1	0	G
GL50803_104685	Caltractin	Axonemal	2	1	0	F in two replicates, G in one replicate
GL50803_137712	Hypothetical protein	Axonemal	0	1	0	G
GL50803_11992	TCP-1 chaperonin sub epsilon	Chaperone	0	2	1	G in two replicates, DSP pre-treatment in one replicate
GL50803_11397	TCP-1 chaperonin subunit beta	Chaperone	0	1	2	G in one replicate, DSP pre-treatment in two replicates
GL50803_16124	TCP-1 chaperonin subunit eta	Chaperone	0	3	0	G
GL50803_13500	TCP-1 chaperonin subunit theta	Chaperone	0	1	2	G in one replicate, DSP pre-treatment in two replicates
GL50803_10231	TCP-1 chaperonin subunit zeta	Chaperone	0	1	2	G in one replicate, DSP pre-treatment in two replicates
GL50803_17482	TCP-1 chaperonin subunit alpha	Chaperone	0	1	2	G in one replicate, DSP pre-treatment in two replicates
GL50803_91919	TCP-1 chaperonin subunit alpha	Chaperone	0	1	2	G in one replicate, DSP pre-treatment in two replicates
GL50803_17411	TCP-1 chaperonin sub gamma	Chaperone	0	2	1	G in two replicates, DSP pre-treatment in one replicate
GL50803_88765	Cytosolic HSP70	Chaperone	0	1	2	G in one replicate, DSP pre-treatment in two replicates

GL50803_17121	Bip	Chaperone	1	2	0	G in two replicates, F in one replicate
GL50803_103891	Chaperonin 60	Chaperone	0	2	0	G
GL50803_9825	TBP-interacting protein TIP49	Nuclear	0	2	0	G
GL50803_17565	TBP-interacting protein TIP49	Nuclear	0	2	1	G in two replicates, DSP pre-treatment in one replicate
GL50803_22850	CMGC MAPK	Signaling	1	3	2	F, G, DSP pre-treatment
GL50803_6430	14-3-3 protein	Signaling	0	1	2	G in one replicate, DSP pre-treatment in two replicates
GL50803_17084	Kinase, NEK	Signaling	0	1	1	G in one replicate, DSP pre-treatment in one replicates
GL50803_14373	Dynamin	Trafficking	3	3	3	F, G, DSP pre-treatment
GL50803_9413	Protein disulfide isomerase PDI2	Trafficking	2	1	0	F in two replicates, G in one replicate
GL50803_15264	hypothetical protein	Hypothetical	0	0	1	DSP pre-treatment
GL50803_17532	Hypothetical protein	Hypothetical	0	0	1	DSP pre-treatment
GL50803_15485	hypothetical protein	Hypothetical	0	1	0	G
GL50803_7807	WD-40 repeat protein	Hypothetical	0	1	0	G
GL50803_137711	Hypothetical protein	Hypothetical	0	1	0	G
GL50803_5859	Hypothetical Protein	Hypothetical	0	1	1	G in one replicate, DSP pre-treatment in one replicate
GL50803_16424	Hypothetical Protein	Hypothetical	1	2	0	G in two replicates, F in one replicate
GL50803_35487	Hypothetical protein	Hypothetical	1	0	0	F
GL50803_37350	Hypothetical protein	Hypothetical	0	1	0	G
GL50803_137739	hypothetical protein	Hypothetical	1	0	0	F
GL50803_137689	Hypothetical protein	Hypothetical	1	0	0	F
GL50803_15251	Hypothetical protein	Hypothetical	1	1	0	G in one replicate, F in one replicate
GL50803_30233	Hypothetical protein	Hypothetical	0	1	0	G

Table S2.3. F-G/Actin interactors which have been previously localized

ORF Number	Identity	Localization
GL50803_2107	Hypothetical	Nuclei
GL50803_13584	Hypothetical	Axonemes
GL50803_14859	Ankyrin repeat	Ventral disc, excluding ventral groove
GL50803_13766	Ankyrin repeat	Ventral disc
GL50803_8692	CBS domain-containing protein	Plasma membrane
GL50803_11380	Hypothetical	Cytoplasm, plasma membrane
GL50803_15918	Hypothetical	Ventral disc, enriched in overlap zone and ventral groove
GL50803_3934	Hypothetical	Anterior and caudal flagella, ventral disc, median body
GL50803_86468	Hypothetical	Inclusions
GL50803_11654	Alpha-1 giardin	Plasma membrane
GL50803_11683	Alpha-3 giardin	Plasma membrane
GL50803_12139	Ankyrin repeat	Ventral disc, lateral crest
GL50803_13273	Dynein intermediate light chain	Flagella
GL50803_15918	Hypothetical	Ventral disc, enriched in overlap zone and ventral groove
GL50803_3934	Hypothetical	Anterior and caudal flagella, ventral disc, median body
GL50803_4410	SALP-1	Ventral disc
GL50803_6171	Hypothetical	Cytoplasm, lateral crest
GL50803_7244	Hypothetical	Cytoplasm, plasma membrane
GL50803_9148	H-SHIPPO-1	Lateral crest, nuclei, median body, flagella
GL50803_94463	Hypothetical	Cytoplasmic
GL50803_86676	Delta-giardin	Ventral disc

Table S2.4. Complete list of proteins identified here as *GI*Actin interactors

Replicate 1				Replicate 2				Replicate			
Protein ID/ORF Number	F Hits	G Hits	DSP Pre-treatment Hits	Protein ID/ORF Number	F Hits	G Hits	DSP Pre-treatment Hits	Protein ID/ORF Number	F Hits	G Hits	DSP Pre-treatment Hits
GL50803_14569	4	21	0	GL50803_112103	397	434	700	GL50803_10016	0	7	7
GL50803_92983	13	15	6	GL50803_112304, GL50803_112312	614	450	508	GL50803_10038	0	4	0
GL50803_24097	10	10	7	GL50803_88765	175	113	424	GL50803_100864	20	9	5
GL50803_2053	0	7	0	GL50803_4812	451	328	412	GL50803_100906	4	20	0
GL50803_24734	7	6	2	GL50803_10311	305	244	395	GL50803_10091	0	13	2
GL50803_11152	0	6	2	GL50803_16453	255	197	373	GL50803_100955	12	6	7
GL50803_11713	3	6	0	GL50803_13608	276	159	305	GL50803_101074	278	214	85
GL50803_14850	0	6	0	GL50803_17570	188	216	305	GL50803_101138	0	14	0
GL50803_10676	7	5	20	GL50803_17121	297	199	294	GL50803_101212	0	2	7
GL50803_3470	3	5	3	GL50803_16125	200	218	271	GL50803_101278	0	82	82
GL50803_2341	5	5	2	GL50803_9909	322	345	254	GL50803_101501	0	10	7
GL50803_8035	0	5	1	GL50803_14373	259	101	254	GL50803_101534	4	15	5
GL50803_14782	3	5	0	GL50803_6687	200	190	192	GL50803_10167	0	35	2
GL50803_16900	0	5	0	GL50803_17230	159	117	192	GL50803_10181	0	6	0
GL50803_14869	0	5	0	GL50803_14285	42	54	192	GL50803_101810	4	4	2
GL50803_137751	14	4	8	GL50803_6430	113	82	186	GL50803_10193	0	7	0
GL50803_115500	9	4	6	GL50803_11043	113	103	181	GL50803_102023	0	14	9
GL50803_10079	8	4	3	GL50803_16867	217	647	158	GL50803_102101	8	73	45
GL50803_27308	2	4	3	GL50803_14521	167	159	153	GL50803_102108	86	135	78
GL50803_16256	1	4	3	GL50803_86511	100	117	153	GL50803_10231	0	13	12
GL50803_104139	2	4	1	GL50803_137617	276	138	147	GL50803_102438	0	4	0
GL50803_13616	2	4	0	GL50803_114609	226	321	147	GL50803_10255	163	74	137
GL50803_89887	2	4	0	GL50803_16833	63	49	147	GL50803_102813	8	12	26
GL50803_2509	1	4	0	GL50803_137716	681	532	141	GL50803_103059	16	31	16
GL50803_8367	1	4	0	GL50803_112079, GL50803_103676	338	293	141	GL50803_10311	278	243	494
GL50803_6185	0	4	0	GL50803_3206	8	9	141	GL50803_103285	0	5	5
GL50803_16833	0	4	0	GL50803_11654	226	110	136	GL50803_103373	90	121	106
GL50803_22455	2	3	3	GL50803_93358	213	270	136	GL50803_103437	0	9	0
GL50803_114192	2	3	1	GL50803_136021, GL50803_101291, GL50803_136020	409	398	130	GL50803_10358	4	39	7
GL50803_23424	0	3	1	GL50803_114787	125	110	130	GL50803_10367	0	20	2
GL50803_7351	3	3	0	GL50803_3331	71	63	130	GL50803_10370	0	3	9
GL50803_2964	2	3	0	GL50803_11118	92	152	124	GL50803_103709	4	19	9
GL50803_7306	1	3	0	GL50803_27925	150	80	119	GL50803_103713	37	44	28
GL50803_17330	0	3	0	GL50803_1345	50	70	119	GL50803_103783	0	10	0
GL50803_11755	0	3	0	GL50803_33769	138	211	113	GL50803_103855	12	3	9
GL50803_34094	0	3	0	GL50803_9779	79	105	113	GL50803_103878	0	3	0
GL50803_2555	0	3	0	GL50803_103373	79	82	113	GL50803_103891	0	11	7
GL50803_14846	0	3	0	GL50803_21942	63	75	113	GL50803_104031	0	2	0
GL50803_28526	0	3	0	GL50803_10822	117	87	107	GL50803_104150	20	1	16
GL50803_8770	0	3	0	GL50803_15383	109	80	107	GL50803_104173	0	56	64
GL50803_9072	0	3	0	GL50803_7532	46	52	102	GL50803_104250	29	7	19
GL50803_10400	0	3	0	GL50803_9704	8	7	102	GL50803_10428	12	20	47
GL50803_15514	0	3	0	GL50803_9848	100	59	96	GL50803_10429	86	35	42
GL50803_16877	0	3	0	GL50803_9827	58	30	96	GL50803_10450	4	13	2
GL50803_10019	1	2	4	GL50803_101074	259	148	90	GL50803_104685	37	11	12
GL50803_113137	4	2	3	GL50803_17395	92	91	90	GL50803_104866	8	9	0
GL50803_6447	3	2	3	GL50803_7195	13	42	90	GL50803_104982	8	0	7
GL50803_17431	1	2	3	GL50803_15297	46	42	85	GL50803_104994	0	3	7
GL50803_113873	1	2	2	GL50803_16844	58	28	79	GL50803_10521	184	64	125
GL50803_94478	0	2	2	GL50803_137618	255	145	73	GL50803_10524	49	14	33

GL50803 30354	0	2	2	GL50803 14938	75	70	73	GL50803 10538	8	34	2
GL50803 17246	0	2	1	GL50803 16387	71	91	73	GL50803 10570	41	25	35
GL50803 13007	0	2	1	GL50803 17163	42	28	73	GL50803 10577	33	34	21
GL50803 33870	2	2	0	GL50803 86676	21	7	73	GL50803 10608	0	4	0
GL50803 11449	1	2	0	GL50803 17055	4	5	73	GL50803 10623	0	19	12
GL50803 7719	1	2	0	GL50803 17153	117	59	68	GL50803 10661	4	27	31
GL50803 17563	1	2	0	GL50803 17090	63	101	68	GL50803 10698	37	11	16
GL50803 17250	1	2	0	GL50803 16588	25	30	68	GL50803 10708	0	13	5
GL50803 93024	0	2	0	GL50803 27310	17	9	68	GL50803 10778	16	2	2
GL50803 105642	0	2	0	GL50803 17063	146	246	62	GL50803 10780	0	5	5
GL50803 90402	0	2	0	GL50803 11359	75	87	62	GL50803 10791	0	1	0
GL50803 14064	0	2	0	GL50803 98054	75	28	62	GL50803 10822	8	42	21
GL50803 29119	0	2	0	GL50803 7204	46	49	62	GL50803 10829	0	10	12
GL50803 15062	0	2	0	GL50803 11354	42	45	62	GL50803 10843	0	31	0
GL50803 5568	0	2	0	GL50803 10524	38	14	62	GL50803 10858	0	3	2
GL50803 5800	0	2	0	GL50803 4410	33	2	62	GL50803 10870	12	14	16
GL50803 17617	0	2	0	GL50803 16322	96	77	56	GL50803 10881	0	2	2
GL50803 23300	0	2	0	GL50803 17547	63	131	56	GL50803 10882	0	6	5
GL50803 13143	0	2	0	GL50803 5810	50	30	56	GL50803 10885	4	64	35
				GL50803_135003, GL50803_135002, GL50803_135001	42	38	56	GL50803 10893	0	5	0
GL50803 3590	0	2	0	GL50803 16667	33	40	56	GL50803 10901	8	2	0
GL50803 5250	0	2	0	GL50803 17060	33	9	56	GL50803 10919	8	1	5
GL50803 14241	0	2	0	GL50803 13747	25	19	56	GL50803 10969	0	2	9
GL50803 11696	0	2	0	GL50803 8826	17	16	56	GL50803 11043	118	120	132
GL50803 137696	0	2	0	GL50803 15410	17	23	56	GL50803 11100	0	1	2
GL50803 10609	0	2	0	GL50803 102813	17	14	56	GL50803 11118	0	159	64
GL50803 21750	0	2	0	GL50803 103709	4	0	56	GL50803 11129	0	4	2
GL50803 6054	0	2	0	GL50803 16412	0	12	56	GL50803 11165	16	15	2
GL50803 17199	0	2	0	GL50803 17244	100	87	51	GL50803 111950	0	11	0
GL50803 14786	4	1	2	GL50803 19436	96	91	51	GL50803 112076	0	54	28
GL50803 9262	2	1	1					GL50803_112079, GL50803_103676	588	656	527
GL50803 96878	2	1	1	GL50803 90872	63	56	51	GL50803 112103	143	475	346
GL50803 7272	0	1	1	GL50803 13864	63	47	51	GL50803 11220	8	2	7
GL50803 4046	0	1	1	GL50803 16717	58	30	51	GL50803_112304, GL50803_112312	682	362	673
GL50803 4838	3	1	0	GL50803 14859	46	9	51	GL50803 112408	0	8	0
GL50803 12807	1	1	0	GL50803 16969	46	28	51	GL50803 11247	4	30	26
GL50803 112633	1	1	0	GL50803 100955	42	35	51	GL50803 112489	0	5	0
GL50803 101832	1	1	0	GL50803 29487	38	28	51	GL50803 112518	4	7	35
GL50803 28232	1	1	0	GL50803 6184	29	21	51	GL50803 112681	4	75	31
GL50803 13622	1	1	0	GL50803 7110	29	28	51	GL50803 112846	8	87	49
GL50803 39212	1	1	0	GL50803 15106	29	49	51	GL50803 11287	33	3	16
GL50803 12089	1	1	0	GL50803 114776	21	21	51	GL50803 112885	0	6	0
GL50803 17379	1	1	0	GL50803 7766	17	35	51	GL50803 11301	53	26	31
GL50803 32398	1	1	0	GL50803 17143	17	21	51	GL50803 11311	20	3	12
GL50803 99743	0	1	0	GL50803 2107	8	2	51	GL50803 113133	4	41	2
GL50803 16430	0	0	2	GL50803 8805	8	0	51	GL50803 113252	0	6	2
GL50803 40747	1	0	1	GL50803 8235	4	16	51	GL50803 113365	0	18	7
GL50803 112017	1	0	1	GL50803 12108	0	26	51	GL50803 113456	4	5	2
GL50803 121061	1	0	1	GL50803 13500	0	59	51	GL50803 11354	29	27	40
GL50803 111732	1	0	1	GL50803 9861	159	143	45	GL50803 113553	45	19	40
GL50803 113439	0	0	1	GL50803 13561	92	115	45	GL50803 113554	0	12	2
GL50803 8512	2	0	0	GL50803 13475	84	143	45	GL50803 11359	4	16	71
GL50803 7891	26	55	16	GL50803 8118	84	82	45	GL50803 113622	20	11	0
GL50803 28234	1	32	3	GL50803 14019	75	77	45	GL50803 113677	4	55	148
GL50803 11448	29	27	40	GL50803 15869	58	61	45	GL50803 113723	12	1	0
GL50803 42377	20	27	7	GL50803 5795	58	84	45				

GL50803 19364	50	12	7	GL50803 5947	42	33	45	GL50803 11380	8	7	24
GL50803 4852	3	12	2	GL50803 15520	42	40	45	GL50803 113876	0	2	0
GL50803 40817	3	40	1	GL50803 16235	25	14	45	GL50803 113892	12	65	9
GL50803 12949	8	30	9	GL50803 14614	25	12	45	GL50803 11390	8	12	28
GL50803 7896	1	10	0	GL50803 93938	21	12	45	GL50803 11397	0	21	7
GL50803 1995	1	10	0	GL50803 9115	13	26	45	GL50803 113987	29	24	2
GL50803 14481	7	54	2	GL50803 113553	13	16	45	GL50803 114119	131	172	141
GL50803 7242	0	18	1	GL50803 14091	75	54	40	GL50803 114210	4	16	5
GL50803 3414	11	9	3	GL50803 7878	50	40	40	GL50803 114246	0	5	0
GL50803 10358	104	352	19	GL50803 7796	46	40	40	GL50803 11434	0	20	7
GL50803 22454	3	8	1	GL50803_121046, GL50803 121045	38	56	40	GL50803_114442, GL50803_112519, GL50803 113284	0	11	2
GL50803 8260	2	8	1	GL50803 11301	29	28	40	GL50803 114497	4	3	7
GL50803 3007	2	8	0	GL50803 3042	13	14	40	GL50803 114498	0	7	2
GL50803 14171	7	37	2	GL50803 22165	4	2	40	GL50803 114546	12	3	7
GL50803 8854	3	79	1	GL50803 12885	4	16	40	GL50803 114609	241	471	424
GL50803 33989	9	7	9	GL50803 9008	0	0	40	GL50803 114626	0	11	0
GL50803 14475	1	7	1	GL50803 10358	205	434	34	GL50803 114776	0	62	42
GL50803 17337	10	76	5	GL50803 14049	50	52	34	GL50803 114777	4	14	0
GL50803 3570	10	30	4	GL50803 12102	46	70	34	GL50803 114787	159	209	137
GL50803 95787	1	12	1	GL50803 10429	38	14	34	GL50803 11486	0	11	16
GL50803 23552	2	6	2	GL50803 11950	38	33	34	GL50803 11495	0	6	0
GL50803 16351	3	6	1	GL50803 17532	25	14	34	GL50803 115052	0	11	0
GL50803 15204	1	6	0	GL50803 7287	13	14	34	GL50803 115129	0	2	0
GL50803 11595	1	6	0	GL50803 8692	8	19	34	GL50803 115159	8	13	0
GL50803 9099	0	6	0	GL50803 17400	8	19	34	GL50803_115338, GL50803 115337	20	7	14
GL50803 10881	0	52	2	GL50803 8560	8	16	34	GL50803 11540	33	42	12
GL50803 7873	15	17	24	GL50803 21505	8	9	34	GL50803_115478, GL50803 115479	29	30	56
GL50803 16435	2	43	0	GL50803 11486	4	14	34	GL50803 11654	229	183	158
GL50803 9825	1	85	1	GL50803 17482	0	30	34	GL50803 11683	73	28	26
GL50803 114121	24	31	29	GL50803 91919	0	16	34	GL50803 11684	106	20	24
GL50803 16652	7	51	2	GL50803 17468	0	0	34	GL50803 11720	29	10	12
GL50803 16867	64	469	31	GL50803 10038	0	0	34	GL50803 11867	12	4	0
GL50803 28696	2	10	0	GL50803 91354	134	38	28	GL50803 11950	65	27	45
GL50803 3147	4	5	3	GL50803 137612	88	54	28	GL50803 11953	16	28	35
GL50803 27266	4	5	2	GL50803 15832	75	171	28	GL50803 11992	4	30	19
GL50803 3563	4	5	2	GL50803 16265	63	56	28	GL50803 12102	94	103	101
GL50803 16805	2	5	1	GL50803 111933	58	35	28	GL50803_121046, GL50803 121045	45	50	45
GL50803 15196	2	5	1	GL50803 111936	58	35	28	GL50803 12108	20	61	35
GL50803 3581	6	5	0	GL50803 15251	58	47	28	GL50803 12139	45	22	16
GL50803 16863	1	5	0	GL50803 7870	50	56	28	GL50803 12150	0	52	33
GL50803 98056	3	34	0	GL50803 480	46	26	28	GL50803 12162	4	9	2
GL50803 112518	0	62	0	GL50803_135231, GL50803 14212	38	33	28	GL50803 12216	163	109	106
GL50803 94658	0	23	0	GL50803 4653	33	9	28	GL50803 12770	12	25	21
GL50803 16659	0	9	0	GL50803 10527	29	14	28	GL50803 12807	0	2	0
GL50803 3036	6	13	2	GL50803 42377	21	5	28	GL50803 12949	0	4	0
GL50803 16310	18	59	6	GL50803 16310	21	30	28	GL50803 12981	12	30	16
GL50803 11342	4	28	8	GL50803 8064	21	30	28	GL50803 12999	41	10	0
GL50803 13988	0	20	1	GL50803 4239	17	12	28	GL50803 13000	8	2	0
GL50803 31671	0	8	1	GL50803 112681	8	14	28	GL50803 13127	0	7	2
GL50803 11170	5	4	5	GL50803 11683	8	12	28	GL50803 13272	0	33	14
GL50803 7007	2	4	2	GL50803 104250	4	5	28	GL50803 13273	12	7	5
GL50803 16715	1	4	2	GL50803 10843	0	16	28	GL50803 13329	4	5	0
GL50803 113219	2	4	1	GL50803 112932	0	5	28	GL50803 13350	0	7	7
GL50803 17371	0	4	1	GL50803 2980	0	14	28	GL50803 13372	8	5	7

GL50803 2452	0	4	1	GL50803 4652	54	30	23	GL50803 13412	0	2	24
GL50803 10361	4	4	0	GL50803 9030	54	38	23	GL50803 13413	0	5	0
GL50803 18471	2	4	0	GL50803 10255	38	12	23	GL50803 1345	0	76	87
GL50803 2013	1	4	0	GL50803 14670	29	21	23	GL50803 13452	4	6	2
GL50803 1383	1	4	0	GL50803 3593	25	38	23	GL50803 13475	0	37	66
GL50803 4578	0	4	0	GL50803 14551	21	28	23	GL50803 13500	0	20	42
GL50803 9363	0	4	0	GL50803 8462	21	26	23	GL50803_135003, GL50803_135002, GL50803_135001	57	30	42
GL50803 17278	0	4	0	GL50803 16824	21	14	23	GL50803_135231, GL50803 14212	37	19	47
GL50803 40244	0	4	0	GL50803 114535	21	7	23	GL50803 13561	135	20	85
GL50803 16322	3	35	13	GL50803 16431	17	35	23	GL50803 13584	20	38	35
GL50803 7843	2	11	3	GL50803 32738	13	7	23	GL50803_135886, GL50803 135885	0	2	2
GL50803 6744	0	11	3	GL50803 113892	13	21	23	GL50803_136021, GL50803_101291, GL50803_136020	604	663	487
GL50803 4156	3	7	1	GL50803 104994	13	7	23	GL50803 13603	0	6	0
GL50803 6917	4	7	0	GL50803 6330	8	5	23	GL50803 13608	86	144	144
GL50803 103944	1	7	0	GL50803 17337	8	12	23	GL50803 13652	4	2	0
GL50803 16067	0	7	0	GL50803 3910	4	5	23	GL50803 13747	8	3	16
GL50803 93550	0	7	0	GL50803 12216	4	7	23	GL50803 137603	0	14	0
GL50803 6564	0	7	0	GL50803 5800	4	7	23	GL50803 137617	265	195	59
GL50803 11953	35	72	35	GL50803 10882	0	2	23	GL50803 137618	253	204	73
GL50803 13127	0	17	0	GL50803 10231	0	5	23	GL50803 137641	0	5	0
GL50803 13652	4	10	2	GL50803 8822	0	0	23	GL50803 13766	12	3	7
GL50803 8001	2	33	0	GL50803 13603	0	2	23	GL50803 137695	0	8	2
GL50803 11247	1	48	1	GL50803 21628	129	103	17	GL50803 137703	4	6	0
GL50803 16390	1	22	0	GL50803 14586	113	38	17	GL50803 137712	0	5	0
GL50803 7204	19	97	14	GL50803 9780	67	45	17	GL50803 137716	196	872	911
GL50803 6242	14	21	4	GL50803 15411	58	35	17	GL50803 13774	8	1	12
GL50803 14712	3	15	0	GL50803 15317	54	52	17	GL50803 137753	24	6	5
GL50803 11034	1	9	0	GL50803 9148	54	7	17	GL50803 13864	78	51	108
GL50803 10450	10	6	10	GL50803 10330	29	16	17	GL50803 13904	0	6	0
GL50803 10530	3	6	1	GL50803 8044	29	7	17	GL50803 13922	20	64	2
GL50803 102444	0	6	1	GL50803 15046	25	33	17	GL50803 13930	20	3	9
GL50803 12150	2	6	0	GL50803 95593	21	14	17	GL50803 13943	8	8	2
GL50803 29252	2	6	0	GL50803 11434	21	12	17	GL50803 13998	0	78	9
GL50803 92031	1	3	3	GL50803 7244	21	16	17	GL50803 14019	8	11	9
GL50803 97130	2	3	1	GL50803 6133	17	19	17	GL50803 14049	12	20	19
GL50803 15304	2	3	1	GL50803 33989	17	7	17	GL50803 14091	45	5	24
GL50803 103891	1	3	1	GL50803 9558	17	9	17	GL50803 14135	0	5	2
GL50803 95530	1	3	1	GL50803 6289	13	12	17	GL50803 14200	4	7	0
GL50803 28699	1	3	1	GL50803 11129	13	5	17	GL50803 14213	0	6	0
GL50803 112870	1	3	1	GL50803 34093	13	9	17	GL50803 14254	29	16	2
GL50803 7522	0	3	1	GL50803 14321	13	23	17	GL50803 14285	16	100	87
GL50803 4928	0	3	1	GL50803 10091	8	33	17	GL50803 14299	8	9	16
GL50803 17578	4	3	0	GL50803_115478, GL50803_115479	8	16	17	GL50803 14311	0	7	2
GL50803 4152	3	3	0	GL50803 5604	8	7	17	GL50803 14321	57	17	35
GL50803 16934	3	3	0	GL50803 9824	8	16	17	GL50803 14329	20	42	56
GL50803 8058	2	3	0	GL50803 15097	8	14	17	GL50803 14373	351	425	285
GL50803 17092	2	3	0	GL50803 17625	4	0	17	GL50803 14375	4	9	5
GL50803 8228	1	3	0	GL50803 10969	4	7	17	GL50803 14404	4	2	5
GL50803 8608	1	3	0	GL50803 14742	4	5	17	GL50803 14460	4	10	7
GL50803 4079	1	3	0	GL50803 10522	4	19	17	GL50803 14469	20	19	14
GL50803 17574	0	3	0	GL50803 12949	4	9	17	GL50803 14481	29	10	16
GL50803 8682	0	3	0	GL50803_27521, GL50803_14256	4	30	17	GL50803 14497	0	5	2

GL50803 6929	0	3	0	GL50803 24321	0	0	17	GL50803 14551	41	33	31
GL50803 17155	0	3	0	GL50803 7323	0	2	17	GL50803 14566	24	0	0
GL50803 16954	0	3	0	GL50803 10361	0	0	17	GL50803 14568	12	7	9
GL50803 9038	0	3	0	GL50803 16353	0	0	17	GL50803 14581	0	9	0
GL50803 16143	0	3	0	GL50803 13756	0	12	17	GL50803 14584	8	8	2
GL50803 39587	0	3	0	GL50803 16802	0	0	17	GL50803 14593	0	7	2
GL50803 17264	5	20	0	GL50803 3209	0	7	17	GL50803 14606	0	8	0
GL50803 104250	15	14	6	GL50803 15409	142	178	11	GL50803 14614	61	35	28
GL50803 113365	0	14	0	GL50803 16086	58	49	11	GL50803 14620	24	54	104
GL50803 14456	3	11	0	GL50803 14622	54	59	11	GL50803 14622	12	23	56
GL50803 15228	36	60	15	GL50803 16343	54	59	11	GL50803 14651	0	3	0
GL50803 16425	11	59	0	GL50803 7891	33	5	11	GL50803 14660	29	11	12
GL50803 39483	2	16	5	GL50803 14620	33	47	11	GL50803 14670	122	20	80
GL50803 102248	8	8	8	GL50803 5845	29	23	11	GL50803 14689	0	4	0
GL50803 14317	1	8	0	GL50803 95162	29	28	11	GL50803 14699	49	27	33
GL50803 101307	0	8	0	GL50803 15427	25	47	11	GL50803 14704	4	7	0
GL50803 7569	4	13	5	GL50803 4606	25	14	11	GL50803 14742	0	7	2
GL50803 135001	31	57	6	GL50803 7999	21	30	11	GL50803 14787	0	5	0
GL50803 22808	4	5	2	GL50803 14660	21	14	11	GL50803 14821	24	6	5
GL50803 5859	2	5	1	GL50803 3582	21	35	11	GL50803 14827	45	22	38
GL50803 6177	0	5	1	GL50803 15127	21	12	11	GL50803 14843	0	1	2
GL50803 7807	0	5	1	GL50803 16124	17	26	11	GL50803 14859	4	40	0
GL50803 11301	48	47	17	GL50803 4590	17	33	11	GL50803 14874	0	7	0
GL50803 21942	5	65	3	GL50803 33862	17	14	11	GL50803 14895	0	4	0
GL50803 17565	2	43	0	GL50803 10829	17	9	11	GL50803 14938	163	97	85
GL50803 15551	29	100	17	GL50803 17193	17	7	11	GL50803 14961	0	4	0
GL50803 31978	6	7	3	GL50803 8245	17	9	11	GL50803 14983	4	2	2
GL50803 29147	4	7	2	GL50803 5333	17	9	11	GL50803 14993	0	18	12
GL50803 12162	2	7	0	GL50803 8407	17	12	11	GL50803 15046	4	39	16
GL50803 21331	0	7	0	GL50803 9810	17	14	11	GL50803 15048	12	14	12
GL50803 5874	0	7	0	GL50803 16716	13	0	11	GL50803 15054	8	5	5
GL50803 14679	0	7	0	GL50803 16424	13	38	11	GL50803 15097	57	89	31
GL50803 12981	19	106	7	GL50803 6022	13	12	11	GL50803 15099	0	14	0
GL50803 3291	3	9	0	GL50803 16768	8	5	11	GL50803 15106	12	78	75
GL50803 15144	1	9	0	GL50803 16477	8	0	11	GL50803 15110	8	2	5
GL50803 86683	0	9	0	GL50803 9355	8	5	11	GL50803 15124	41	5	21
GL50803 14660	9	20	4	GL50803 11247	8	16	11	GL50803 15148	0	16	7
GL50803 16404	8	11	2	GL50803 10016	8	5	11	GL50803 15196	0	3	0
GL50803 11950	5	43	2	GL50803 5517	8	9	11	GL50803 15204	24	8	12
GL50803 16387	15	92	8	GL50803 15918	8	9	11	GL50803 15213	8	10	7
GL50803 19003	5	36	6	GL50803 4463	8	12	11	GL50803 15214	8	7	14
GL50803 7244	26	79	2	GL50803 9062	8	35	11	GL50803 15215	49	7	14
GL50803 104685	8	31	5	GL50803 20195	8	2	11	GL50803 15218	12	22	2
GL50803 4547	9	48	4	GL50803 3978	4	2	11	GL50803 15228	65	14	38
GL50803 15054	9	40	0	GL50803 4349	4	7	11	GL50803 15247	24	10	19
GL50803 113554	1	8	0	GL50803 15099	4	5	11	GL50803 15252	0	11	2
GL50803 16929	0	8	0	GL50803 7439	4	12	11	GL50803 15260	33	12	28
GL50803 3417	0	8	0	GL50803 16044	4	12	11	GL50803 15297	61	35	64
GL50803 7375	0	8	0	GL50803 21118	4	7	11	GL50803 15307	0	5	0
GL50803 14299	7	6	1	GL50803 27059	4	5	11	GL50803 15317	0	13	2
GL50803 15454	2	6	0	GL50803 104866	4	2	11	GL50803 15383	16	17	21
GL50803 91398	7	4	1	GL50803 14993	4	0	11	GL50803 15397	29	7	14
GL50803 10908	4	4	1	GL50803 7260	4	2	11	GL50803 15398	4	12	2
GL50803 28657	2	4	1	GL50803 23634	4	0	11	GL50803 15409	98	76	68
GL50803 21803	1	4	1	GL50803 14299	4	2	11	GL50803 15410	12	9	9
GL50803 11976	0	4	1	GL50803 39483	4	2	11	GL50803 15411	4	33	33
GL50803 15474	0	4	1	GL50803 17585	0	2	11	GL50803 15427	0	24	16
GL50803 3032	0	4	1	GL50803 33218	0	5	11	GL50803 15454	0	10	0
GL50803 11246	1	4	0	GL50803 9088	0	0	11	GL50803 15487	0	6	2

GL50803 16613	0	4	0	GL50803 101810	0	0	11	GL50803 15495	0	3	0
GL50803 114885	0	4	0	GL50803 10661	0	5	11	GL50803 15499	8	13	0
GL50803 137609	0	4	0	GL50803 17472	0	0	11	GL50803 15520	122	31	73
GL50803 32741	0	4	0	GL50803 17000	0	7	11	GL50803 15551	16	14	16
GL50803 37449	0	2	4	GL50803 16521	0	2	11	GL50803 15567	4	8	7
GL50803 97075	0	2	3	GL50803 17254	0	5	11	GL50803 15591	45	29	49
GL50803 16534	0	2	3	GL50803 27658	0	0	11	GL50803 15600	0	4	0
GL50803 15847	0	2	2	GL50803 28234	0	2	11	GL50803 15823	0	6	0
GL50803 7373	0	2	2	GL50803 11992	0	21	11	GL50803 15832	0	80	19
GL50803 25238	2	2	1	GL50803 14497	0	7	11	GL50803 15847	4	8	0
GL50803 14322	1	2	1	GL50803 22677	0	2	11	GL50803 15869	45	54	52
GL50803 7871	0	2	1	GL50803 14374	0	0	11	GL50803 15871	8	18	5
GL50803 14765	0	2	1	GL50803 16745	117	73	6	GL50803 15918	78	13	26
GL50803 16227	0	2	1	GL50803 8854	50	218	6	GL50803 15954	0	5	0
GL50803 37488	2	2	0	GL50803 137606	46	21	6	GL50803 15956	0	12	2
GL50803 17586	2	2	0	GL50803 9183	46	218	6	GL50803 15983	0	16	0
GL50803 8003	1	2	0	GL50803 13584	38	16	6	GL50803 16039	0	3	0
GL50803 8049	1	2	0	GL50803 15228	38	28	6	GL50803 16044	53	20	31
GL50803 33030	1	2	0	GL50803 16779	38	23	6	GL50803 16067	12	9	12
GL50803 9523	0	2	0	GL50803 14699	33	30	6	GL50803 16076,			
GL50803 13164	0	2	0	GL50803 4547	33	26	6	GL50803 14521	257	171	144
GL50803 14950	0	2	0	GL50803 16160	33	33	6	GL50803 16085	16	0	7
GL50803 8160	0	2	0	GL50803 10428	29	38	6	GL50803 16086	0	7	24
GL50803 10901	0	2	0	GL50803 40817	29	19	6	GL50803 16114	12	8	5
GL50803 6531	0	2	0	GL50803 11540	25	9	6	GL50803 16124	8	61	56
GL50803 7790	0	2	0	GL50803 113987	25	2	6	GL50803 16125	29	243	238
GL50803 8886	0	2	0	GL50803 14874	21	16	6	GL50803 16200	0	3	0
GL50803 33862	27	78	20	GL50803 13268	21	28	6	GL50803 16202	20	26	2
GL50803 5593	18	52	10	GL50803 15124	17	14	6	GL50803 16235	90	44	75
GL50803 3538	13	19	7	GL50803 5593	17	7	6	GL50803 16237	0	7	0
GL50803 23888	20	62	29	GL50803 14329	17	47	6	GL50803 16265	135	63	137
GL50803 4507	2	26	0	GL50803 36069	13	16	6	GL50803 16267	0	3	0
GL50803 10661	4	13	0	GL50803 24319	13	14	6	GL50803 16279	4	0	2
GL50803 15520	11	63	0	GL50803 97219	13	30	6	GL50803 16310	53	33	40
GL50803 135231	20	61	11	GL50803 16114	13	14	6	GL50803 16312	0	27	2
GL50803 15410	3	33	5	GL50803 15260	13	9	6	GL50803 16317	0	9	0
GL50803 114497	2	20	1	GL50803 113622	13	0	6	GL50803 16322	0	12	5
GL50803 16263	13	18	11	GL50803 10623	13	14	6	GL50803 16328	0	2	0
GL50803 15546	2	9	4	GL50803 137719	13	7	6	GL50803 16342	0	4	0
GL50803 3910	5	9	1	GL50803 17568	13	7	6	GL50803 16343	0	33	16
GL50803 39312	0	9	1	GL50803 11684	13	38	6	GL50803 16353	12	49	12
GL50803 16313	0	9	0	GL50803 13000	13	5	6	GL50803 16368	0	5	0
GL50803 14827	0	34	0	GL50803 27717	13	2	6	GL50803 16371	0	3	12
GL50803 15046	16	73	15	GL50803 9515	13	16	6	GL50803 16376	49	30	40
GL50803 5942	0	23	0	GL50803 14171	8	30	6	GL50803 16387	16	29	31
GL50803 7789	6	30	1	GL50803 3861	8	14	6	GL50803 16390	0	17	2
GL50803 121045	40	97	20	GL50803 8559	8	2	6	GL50803 16404	49	13	7
GL50803 16265	6	67	3	GL50803 23004	8	5	6	GL50803 16412	0	77	26
GL50803 14329	16	84	3	GL50803 8377	8	30	6	GL50803 16424	12	12	5
GL50803 34179	1	7	1	GL50803 5883	8	70	6	GL50803 16431	16	20	49
GL50803 15538	1	7	0	GL50803 6564	8	0	6	GL50803 16443	8	7	5
GL50803 9327	1	7	0	GL50803 3256	8	5	6	GL50803 16453	224	272	372
GL50803 14699	10	57	7	GL50803 8741	8	14	6	GL50803 1649	16	6	12
GL50803 27918	13	24	11	GL50803 7789	8	7	6	GL50803 16507	12	53	5
GL50803 10428	1	37	1	GL50803 15551	8	23	6	GL50803 16521	0	2	5
GL50803 10813	11	5	8	GL50803 10422	8	21	6	GL50803 16522	8	11	12
GL50803 16966	1	5	0	GL50803 23833	8	5	6	GL50803 16525	159	125	224
GL50803 17106	0	5	0	GL50803 19003	8	21	6	GL50803 16540	0	5	0
								GL50803 16549	4	13	16

GL50803 9779	29	101	28	GL50803 17150	8	7	6	GL50803 16567	0	24	2
GL50803 6430	28	111	20	GL50803 12139	8	5	6	GL50803 16568	0	6	5
GL50803 16114	8	26	6	GL50803 10450	8	16	6	GL50803 16572	0	3	0
GL50803 15427	3	26	0	GL50803 12981	8	23	6	GL50803 16575	0	13	0
GL50803 10780	7	13	7	GL50803 23064	4	5	6	GL50803 16588	163	28	118
GL50803 16044	12	56	14	GL50803 103713	4	7	6	GL50803 16592	0	16	0
GL50803 14460	0	16	0	GL50803 7953	4	5	6	GL50803 16602	0	3	0
GL50803 137711	2	8	4	GL50803 137703	4	2	6	GL50803 16636	16	5	5
GL50803 11043	14	94	10	GL50803 14566	4	5	6	GL50803 16648	12	20	0
GL50803 42000	5	11	3	GL50803 112673, GL50803 112135	4	7	6	GL50803 16652	78	20	47
GL50803 7962	0	11	0	GL50803 103855	4	2	6	GL50803 16653	37	25	33
GL50803 9803	3	14	0	GL50803 8394	4	2	6	GL50803 16659	0	5	2
GL50803 11359	1	79	6	GL50803 103783	4	9	6	GL50803 16667	0	147	42
GL50803 8118	12	112	17	GL50803 24880	4	0	6	GL50803 16717	12	33	24
GL50803 17364	7	26	6	GL50803 24947	4	5	6	GL50803 16720	0	27	97
GL50803 15106	10	56	1	GL50803 6367	4	14	6	GL50803 16745	12	42	141
GL50803 87149	7	6	8	GL50803 95880	4	0	6	GL50803 16766	0	14	2
GL50803 2012	2	6	4	GL50803 22855	4	2	6	GL50803 16768	0	8	0
GL50803 21943	5	6	2	GL50803 10919	4	5	6	GL50803 16773	0	5	0
GL50803 5186	0	6	2	GL50803 115066	4	9	6	GL50803 16802	4	8	12
GL50803 95162	1	6	1	GL50803 17224	4	9	6	GL50803 16804	29	33	9
GL50803 14961	1	6	1	GL50803 6226	4	12	6	GL50803 16811	0	3	2
GL50803 19378	1	3	2	GL50803 1649	4	0	6	GL50803 16817	12	12	7
GL50803 16544	4	3	1	GL50803 5859	4	0	6	GL50803 16824	37	48	49
GL50803 90950	1	3	1	GL50803 27918	4	5	6	GL50803 16833	0	12	7
GL50803 114201	1	3	1	GL50803 112114	4	9	6	GL50803 16843	0	2	0
GL50803 5328	3	3	0	GL50803 7843	4	2	6	GL50803 16844	106	21	40
GL50803 9493	0	3	0	GL50803 17622	4	2	6	GL50803 16867	8	163	118
GL50803 17347	0	3	0	GL50803 36168	4	2	6	GL50803 16887	0	2	0
GL50803 10875	0	3	0	GL50803 7082	4	7	6	GL50803 16888	0	2	0
GL50803 115337	0	3	0	GL50803 10870	0	0	6	GL50803 16891	0	5	0
GL50803 2007	0	3	0	GL50803 23888	0	7	6	GL50803 16906	0	4	0
GL50803 6048	0	3	0	GL50803 29490	0	5	6	GL50803 16916	0	4	0
GL50803 16034	0	3	0	GL50803 4507	0	2	6	GL50803 16929	0	5	0
GL50803 34058	0	3	0	GL50803 10196	0	2	6	GL50803 16935	20	1	14
GL50803 16371	0	3	0	GL50803 6439	0	0	6	GL50803 16945	8	6	2
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GL50803 7999	12	87	3	GL50803 16504	0	0	6	GL50803 1695	0	4	0
GL50803 14584	2	22	0	GL50803 2012	0	2	6	GL50803 16954	0	2	19
GL50803 27310	1	16	0	GL50803 9367	0	0	6	GL50803 16967	0	15	2
GL50803 13475	7	52	4	GL50803 27308	0	0	6	GL50803 16969	0	8	5
GL50803 13273	0	10	0	GL50803 14704	0	0	6	GL50803 16975	0	3	5
GL50803 16424	0	10	0	GL50803 16818	0	0	6	GL50803 16993	0	0	7
GL50803 9489	0	10	0	GL50803 8903	0	26	6	GL50803 16996	65	16	19
GL50803 7870	36	104	32	GL50803 14547	0	0	6	GL50803 17025	4	4	0
GL50803 15099	2	24	3	GL50803 39612	0	0	6	GL50803 17046	37	11	9
GL50803 137748	3	7	2	GL50803 4259	0	0	6	GL50803 17053	0	3	2
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GL50803 17395	0	62	2	GL50803 15495	0	0	6	GL50803 17055	4	0	19
GL50803 16412	2	11	1	GL50803 10778	0	2	6	GL50803 17056	78	31	54
GL50803 5947	36	83	26	GL50803 14481	0	5	6	GL50803 17060	8	54	12
GL50803 7110	17	23	8	GL50803 13903	0	0	6	GL50803 17063	90	207	207
GL50803 14019	12	54	17	GL50803 17223	0	2	6	GL50803 17084	0	2	5
GL50803 112103	59	209	11	GL50803 2452	0	2	6	GL50803 17090	33	57	21
GL50803 3331	7	32	8	GL50803 6242	0	2	6	GL50803 17106	0	7	0
GL50803 8745	0	12	1	GL50803 11436	0	0	6	GL50803 17110	0	8	2
GL50803 13603	2	8	0	GL50803 4946	0	2	6	GL50803 17121	151	327	186
GL50803 37985	1	4	1	GL50803 14539	0	0	6	GL50803 17132	0	11	0

GL50803 91324	0	4	1	GL50803 17264	0	2	6	GL50803 17143	0	16	31
GL50803 24662	0	4	1	GL50803 17451	0	0	6	GL50803 17150	4	6	0
GL50803 88960	4	4	0	GL50803 86683	0	2	6	GL50803 17153	204	126	174
GL50803 28549	3	4	0	GL50803 33166	0	14	6	GL50803 17163	73	32	19
GL50803 14390	1	4	0	GL50803 115239	0	0	6	GL50803 17165	0	8	0
GL50803 10341	0	4	0	GL50803 5786	0	0	6	GL50803 17224	37	2	5
GL50803 10868	0	4	0	GL50803 14712	0	0	6	GL50803 17230	404	190	134
GL50803 17110	0	4	0	GL50803 12150	0	5	6	GL50803 17231	0	3	2
GL50803 14256	19	61	9	GL50803 16161	0	0	6	GL50803 17243	0	48	0
GL50803 16453	71	127	49	GL50803 10324	0	0	6	GL50803 17244	94	55	89
GL50803 15260	9	33	5	GL50803 15148	0	0	6	GL50803 17246	8	1	0
GL50803 14620	3	63	5	GL50803 31402	0	0	6	GL50803 17249	0	76	64
GL50803 16431	3	47	1	GL50803 17558	0	0	6	GL50803 17254	0	28	12
GL50803 21118	1	26	0	GL50803 15214	0	7	6	GL50803 17255	24	19	9
GL50803 3256	11	27	7	GL50803 113655	0	0	6	GL50803 17265	16	78	7
GL50803 91643	7	27	0	GL50803 15397	0	0	6	GL50803 17277	12	7	0
GL50803 10330	0	14	7	GL50803 17130	0	0	6	GL50803 17304	16	16	9
GL50803 6724	7	10	3	GL50803 4838	0	0	6	GL50803 17327	4	91	66
GL50803 91504	2	5	2	GL50803 17411	0	28	6	GL50803 17332	4	38	24
GL50803 90710	1	5	1	GL50803 87422	0	2	6	GL50803 17337	4	4	7
GL50803 14689	0	5	0	GL50803 11397	0	0	6	GL50803 17362	0	3	0
GL50803 7030	0	5	0	GL50803 9503	0	0	6	GL50803 17364	12	4	14
GL50803 7195	11	42	1	GL50803 4043	0	0	6	GL50803 17393	0	4	0
GL50803 7588	2	16	1	GL50803 13273	0	2	6	GL50803 17395	57	52	137
GL50803 14049	24	75	17	GL50803 15048	0	0	6	GL50803 17400	65	26	59
GL50803 13268	22	59	30	GL50803 4059	0	2	6	GL50803 17406	0	15	0
GL50803 16720	1	11	0	GL50803 22850	0	5	6	GL50803 17411	4	22	33
GL50803 17060	14	47	0	GL50803 137708, GL50803 137707, GL50803 41472	0	0	6	GL50803 17413	0	1	2
GL50803 101291	165	502	54	GL50803 13930	0	2	6	GL50803 17430	0	5	9
GL50803 13747	14	24	4	GL50803 15218	0	0	6	GL50803 17432	0	33	26
GL50803 9115	3	6	0	GL50803 134441, GL50803 134442	0	2	6	GL50803 17448	0	4	2
GL50803 11100	3	6	0	GL50803 2834	0	0	6	GL50803 17460	8	3	5
GL50803 137684	2	6	0	GL50803 8528	0	0	6	GL50803 17482	0	34	19
GL50803 102710	0	6	0	GL50803 14213	0	2	6	GL50803 17520	0	9	0
GL50803 7878	35	110	53	GL50803 16006	0	0	6	GL50803 17547	175	192	334
GL50803 13904	3	8	2	GL50803 21750	0	2	6	GL50803 17551	0	6	5
GL50803 27614	2	8	2	GL50803 16227	0	0	6	GL50803 17558	8	1	0
GL50803 102023	1	8	0	GL50803 16869	0	0	6	GL50803 17565	0	33	2
GL50803 6687	48	128	59	GL50803 13890	0	0	6	GL50803 17566	12	1	12
GL50803 5604	14	43	27	GL50803 14345	0	0	6	GL50803 17567	16	7	12
GL50803 103709	3	9	0	GL50803 17255	0	2	6	GL50803 17568	4	7	5
GL50803 8426	2	9	0	GL50803 15514	0	2	6	GL50803 17570	188	200	334
GL50803 26364	1	9	0	GL50803 16135	0	2	6	GL50803 17571	0	4	0
GL50803 8462	22	120	54	GL50803 11034	0	2	6	GL50803 17579	0	2	2
GL50803 6133	13	37	12	GL50803 15264	0	0	6	GL50803 17585	0	7	5
GL50803 10919	13	61	46	GL50803 17304	0	0	6	GL50803 17625	4	5	7
GL50803 13412	0	11	1	GL50803 15252	0	2	6	GL50803 17627	4	2	0
GL50803 3896	6	12	5	GL50803 41212	129	253	0	GL50803 18470	4	18	0
GL50803 15127	12	12	3	GL50803 113677	125	169	0	GL50803 19003	0	5	0
GL50803 33166	0	12	1	GL50803 17249	104	131	0	GL50803 19436	78	89	146
GL50803 34093	11	26	32	GL50803 10167	104	199	0	GL50803 2012	8	1	0
GL50803 2198	1	13	6	GL50803 7444	71	77	0	GL50803 2053	4	3	2
GL50803 3595	1	13	1	GL50803 5375	58	42	0	GL50803 2098	0	4	2
GL50803 17056	9	86	16	GL50803 11390	54	30	0	GL50803 2107	61	17	9
GL50803 5810	47	45	35	GL50803 96670	42	9	0	GL50803 21118	20	52	14
GL50803 9183	11	228	0	GL50803 6464	38	12	0	GL50803 21331	0	5	0

GL50803 17150	20	16	14	GL50803 11311	33	16	0	GL50803 21411	20	33	0
GL50803 16368	16	49	27	GL50803 9413	29	14	0	GL50803 21423	12	23	24
GL50803 14521	91	206	98	GL50803 16380	29	12	0	GL50803 21444	0	1	2
GL50803 29487	3	35	1	GL50803 102108	29	7	0	GL50803 21474	4	2	2
GL50803 17163	77	74	56	GL50803 102101	29	14	0	GL50803 21505	0	33	19
GL50803 17054	1	61	3	GL50803 17327	25	80	0	GL50803 21628	204	123	344
GL50803 36069	9	63	23	GL50803 16525	25	42	0	GL50803 21750	0	1	5
GL50803 15983	2	32	1	GL50803 14827	21	16	0	GL50803 21942	0	266	104
GL50803 29327	10	30	12	GL50803 2933	21	21	0	GL50803 22204	4	12	2
GL50803 8377	1	19	2	GL50803 101278	21	73	0	GL50803 22543	0	28	9
GL50803 16202	2	17	0	GL50803 6135	21	14	0	GL50803 22677	0	6	0
GL50803 8559	14	10	6	GL50803 9808	21	45	0	GL50803 22808	0	29	7
GL50803 137695	0	10	1	GL50803 32999	21	19	0	GL50803 22850	12	7	12
GL50803 111950	7	9	3	GL50803 10367	21	12	0	GL50803 23004	4	5	5
GL50803 6752	7	8	7	GL50803 112846	21	14	0	GL50803 23017	4	7	12
GL50803 16298	0	8	2	GL50803 12091	17	2	0	GL50803 2323	0	2	2
GL50803 4954	0	6	2	GL50803 8444	17	2	0	GL50803 23300	16	0	7
GL50803 16456	1	6	1	GL50803 16652	17	16	0	GL50803 23552	16	3	7
GL50803 14346	1	6	0	GL50803 17551	17	38	0	GL50803 23767	4	1	2
GL50803 5890	3	5	6	GL50803 94463	17	12	0	GL50803 23833	102	69	73
GL50803 15252	10	5	5	GL50803 93294	17	30	0	GL50803 23888	4	6	0
GL50803 35180	3	5	3	GL50803 24451	13	0	0	GL50803 24321	0	8	0
GL50803 21662	3	5	1	GL50803 8726	13	0	0	GL50803 24425	8	4	7
GL50803 8616	0	5	1	GL50803 6171	13	7	0	GL50803 24451	53	4	16
GL50803 15376	0	5	0	GL50803 9719	13	84	0	GL50803 2452	0	5	9
GL50803 9196	0	5	0	GL50803 2825	8	5	0	GL50803 24537	0	19	0
GL50803 15485	2	4	1	GL50803 11287	8	12	0	GL50803 24662	0	5	0
GL50803 3475	2	4	1	GL50803 9803	8	5	0	GL50803 24842	20	21	19
GL50803 27326	0	4	1	GL50803 137726	8	2	0	GL50803 24880	8	13	0
GL50803 17333	0	4	1	GL50803 16263	8	5	0	GL50803 25205	0	4	0
GL50803 17046	2	4	0	GL50803 17277	8	19	0	GL50803 2562	20	10	9
GL50803 3978	2	4	0	GL50803 14270	8	2	0	GL50803 26199	12	7	14
GL50803 13272	0	4	0	GL50803 16648	8	9	0	GL50803 26364	8	5	12
GL50803 16264	0	4	0	GL50803 17195	8	2	0	GL50803 27059	0	5	0
GL50803 15149	2	3	2	GL50803 92760	8	49	0	GL50803 27266	33	6	19
GL50803 113094	2	3	2	GL50803 10780	8	5	0	GL50803 27310	4	27	31
GL50803 38462	1	3	2	GL50803 23496	8	2	0	GL50803_27521, GL50803 14256	12	13	12
GL50803 14457	0	3	2	GL50803 16368	8	14	0	GL50803 27614	0	4	0
GL50803 12999	5	3	1	GL50803 5744	8	5	0	GL50803 27717	24	20	2
GL50803 12830	5	3	1	GL50803 7030	8	2	0	GL50803 27925	33	46	85
GL50803 17111	2	3	1	GL50803_135886, GL50803 135885	8	2	0	GL50803 28234	4	0	12
GL50803 34701	1	3	1	GL50803 17054	8	9	0	GL50803 2825	24	10	14
GL50803 16761	0	3	1	GL50803 7188	8	12	0	GL50803 28336	0	3	0
GL50803 14661	0	3	1	GL50803 17056	8	16	0	GL50803 28379	0	7	0
GL50803 16729	3	3	0	GL50803 8794	8	14	0	GL50803 28699	0	5	0
GL50803 10825	1	3	0	GL50803 137744	8	2	0	GL50803 29307	0	6	0
GL50803 15192	1	3	0	GL50803 7031	8	5	0	GL50803 29327	16	3	9
GL50803 16760	0	3	0	GL50803 98056	8	23	0	GL50803 2933	8	3	0
GL50803 36168	2	2	6	GL50803 10423	8	0	0	GL50803 29487	4	21	33
GL50803 11244	3	2	2	GL50803 10521	8	7	0	GL50803 2970	12	8	9
GL50803 15190	1	2	1	GL50803 8713	8	0	0	GL50803 2980	0	2	2
GL50803 21232	1	2	1	GL50803 14606	8	7	0	GL50803 3007	0	3	0
GL50803 14608	0	2	1	GL50803 1894	4	5	0	GL50803 3032	12	5	5
GL50803 16568	0	2	1	GL50803 12598	4	12	0	GL50803 3042	0	18	5
GL50803 137746	5	2	0	GL50803 4039	4	0	0	GL50803 30476	4	11	0
GL50803 5871	2	2	0	GL50803 21474	4	0	0	GL50803 3206	12	18	26
GL50803 8172	2	2	0	GL50803 6131	4	0	0	GL50803 3256	0	7	5

GL50803 2011	1	2	0	GL50803 17011	4	5	0	GL50803 32658	0	30	2
GL50803 24043	1	2	0	GL50803 137739	4	0	0	GL50803 32681	0	1	2
GL50803 16373	1	2	0	GL50803 3934	4	0	0	GL50803 32697	0	3	0
GL50803 4331	0	2	0	GL50803 17332	4	0	0	GL50803 32778	8	14	7
GL50803 2661	0	2	0	GL50803 12999	4	2	0	GL50803 3313	4	2	7
GL50803 11384	0	2	0	GL50803 6724	4	0	0	GL50803 33166	4	9	9
GL50803 14174	0	2	0	GL50803 9173	4	7	0	GL50803 3331	49	85	68
GL50803 10269	0	2	0	GL50803 35487	4	0	0	GL50803 33685	4	4	5
GL50803 114216	0	2	0	GL50803 6812	4	5	0	GL50803 33689	0	34	14
GL50803 16658	0	2	0	GL50803 114626	4	0	0	GL50803 33769	16	145	87
GL50803 21082	0	2	0	GL50803 113133	4	7	0	GL50803 33862	53	14	21
GL50803 6757	0	2	0	GL50803 13412	4	9	0	GL50803 33989	20	2	12
GL50803 16450	0	2	0	GL50803 21048	4	0	0	GL50803 3409	0	7	0
GL50803 31309	0	2	0	GL50803 112063	4	0	0	GL50803 34179	0	2	2
GL50803 17389	0	2	0	GL50803 8496	4	7	0	GL50803 35428	0	19	2
GL50803 24133	1	1	3	GL50803 14469	4	5	0	GL50803 3582	0	2	0
GL50803 12741	2	1	2	GL50803 16507	4	16	0	GL50803 3593	0	48	40
GL50803 13282	1	1	2	GL50803 15139	4	2	0	GL50803 36069	0	14	12
GL50803 221692	0	1	2	GL50803 17342	4	0	0	GL50803 3643	0	26	9
GL50803 32606	3	1	1	GL50803 115159	4	7	0	GL50803 3678	20	9	16
GL50803 101194	2	1	1	GL50803 17432	4	9	0	GL50803 37452	4	3	0
GL50803 17119	1	1	1	GL50803 6752	4	0	0	GL50803 37907	4	2	2
GL50803 9718	1	1	1	GL50803 24979	4	0	0	GL50803 37985	0	6	0
GL50803 11613	0	1	1	GL50803 35066	4	0	0	GL50803 3822	0	34	12
GL50803 93103	0	1	1	GL50803 24537	4	9	0	GL50803 3861	0	35	9
GL50803 22112	0	1	1	GL50803 33685	4	2	0	GL50803 3869	0	2	0
GL50803 16526	0	1	1	GL50803 93548	4	28	0	GL50803 3910	29	8	19
GL50803 9079	2	1	0	GL50803 14457	4	5	0	GL50803 39312	0	2	0
GL50803 15049	2	1	0	GL50803 100864	4	7	0	GL50803 3934	57	8	26
GL50803 13346	1	1	0	GL50803 98985	4	2	0	GL50803 40067	0	4	0
GL50803 16975	1	1	0	GL50803 10885	4	12	0	GL50803 40228	0	2	5
GL50803 16111	1	1	0	GL50803 16839	4	0	0	GL50803 4026	0	6	0
GL50803 137705	1	1	0	GL50803 7268	4	0	0	GL50803 4044	0	6	0
GL50803 21502	1	1	0	GL50803 7349	4	2	0	GL50803 40496	0	64	2
GL50803 8210	1	1	0	GL50803 5250	4	0	0	GL50803 40521	0	5	5
GL50803 3993	1	1	0	GL50803 105915	4	0	0	GL50803 40817	4	22	7
GL50803 20782	0	1	0	GL50803 17364	4	12	0	GL50803 41212	0	27	73
GL50803 92739	0	1	0	GL50803 2622	4	0	0	GL50803 4149	0	11	0
GL50803 10840	0	1	0	GL50803 14460	4	0	0	GL50803 41512	24	49	16
GL50803 15867	0	1	0	GL50803 137603	4	0	0	GL50803 4197	0	6	0
GL50803 3367	0	1	0	GL50803 14895	4	0	0	GL50803 4204	0	3	0
GL50803 17261	0	1	0	GL50803 113213	4	2	0	GL50803 4239	57	10	28
GL50803 89535	0	1	0	GL50803 11740	4	0	0	GL50803 4248	0	2	0
GL50803 15399	0	1	0	GL50803 114777	4	0	0	GL50803 4331	0	3	0
GL50803 10802	0	1	0	GL50803 15591	4	0	0	GL50803 4349	0	3	7
GL50803 5567	0	1	0	GL50803 21423	4	0	0	GL50803 4365	0	5	0
GL50803 7723	0	1	0	GL50803 4383	4	0	0	GL50803 4410	180	65	75
GL50803 14602	0	1	0	GL50803 21398	4	2	0	GL50803 4463	4	11	0
GL50803 13776	0	1	0	GL50803 116477	4	0	0	GL50803 4507	0	4	0
GL50803 6026	0	1	0	GL50803 7242	4	12	0	GL50803 4547	110	36	45
GL50803 37711	0	1	0	GL50803 9196	4	0	0	GL50803 4627	4	12	2
GL50803 86680	0	1	0	GL50803 11441	4	2	0	GL50803 4652	29	41	28
GL50803 40995	0	1	0	GL50803 32658	4	0	0	GL50803 4653	0	17	5
GL50803 14436	0	1	0	GL50803 40521	4	0	0	GL50803 480	41	27	24
GL50803 16906	0	1	0	GL50803 104685	4	0	0	GL50803 4812	571	308	231
GL50803 8819	0	1	0	GL50803 7414	4	2	0	GL50803 5010	20	10	7
GL50803 14993	0	1	0	GL50803 15011	4	2	0	GL50803 5161	4	2	2
GL50803 95661	0	1	0	GL50803 7778	4	2	0	GL50803 5188	0	9	0
GL50803 13876	0	1	0	GL50803 36036	4	0	0	GL50803 5328	4	3	2

GL50803 35670	0	1	0	GL50803 2970	4	30	0	GL50803 5333	57	9	28
GL50803 9861	11	188	20	GL50803 14921	4	9	0	GL50803 5346	0	1	2
GL50803 33769	21	129	18	GL50803 86440	4	0	0	GL50803 5359	12	42	19
GL50803 17244	7	86	10	GL50803 17164	4	0	0	GL50803 5375	20	4	14
GL50803 7082	15	63	27	GL50803 1383	4	0	0	GL50803 5593	106	32	54
GL50803 10231	5	52	3	GL50803 22291	4	2	0	GL50803 5604	8	5	7
GL50803 7766	0	41	8	GL50803 5785	4	0	0	GL50803 5649	4	24	16
GL50803 6563	5	24	1	GL50803 3349	0	9	0	GL50803 5659	0	2	0
GL50803 10091	23	91	72	GL50803 137727	0	2	0	GL50803 5744	33	6	12
GL50803 5517	5	21	12	GL50803 3007	0	2	0	GL50803 5768	0	2	0
GL50803 3593	9	34	1	GL50803 16298	0	2	0	GL50803 5785	0	6	0
GL50803 4349	6	16	6	GL50803 15455	0	19	0	GL50803 5795	0	45	2
GL50803 91919	8	74	3	GL50803 11521	0	5	0	GL50803 5800	0	7	14
GL50803 17153	19	102	20	GL50803 112633	0	2	0	GL50803 5809	4	18	0
GL50803 21628	13	122	25	GL50803 8002	0	5	0	GL50803 5810	90	19	52
GL50803 17327	6	90	8	GL50803 15451	0	5	0	GL50803 5845	86	48	80
GL50803 16086	1	37	9	GL50803 15134	0	2	0	GL50803 5890	4	2	2
GL50803 112681	4	35	0	GL50803 101534	0	2	0	GL50803 5942	16	32	24
GL50803 103676	81	270	31	GL50803 22808	0	5	0	GL50803 5947	94	39	64
GL50803 11118	16	91	17	GL50803 17516	0	2	0	GL50803 6022	0	18	5
GL50803 11992	2	60	0	GL50803 33030	0	2	0	GL50803 6026	0	1	2
GL50803 23004	5	38	19	GL50803 3538	0	5	0	GL50803 6133	0	37	12
GL50803 16525	3	74	1	GL50803 7552	0	5	0	GL50803 6135	0	21	7
GL50803 9088	6	9	8	GL50803 10813	0	2	0	GL50803 6148	0	3	0
GL50803 16443	2	16	0	GL50803 14048	0	5	0	GL50803 6171	0	4	12
GL50803 134441	6	8	5	GL50803_114442, GL50803_112519, GL50803_113284, GL50803 92983	0	2	0	GL50803 6184	29	23	24
GL50803 8917	3	15	0	GL50803 102542	0	5	0	GL50803 6226	0	63	7
GL50803 97219	0	37	3	GL50803 17393	0	12	0	GL50803 6242	24	13	9
GL50803 4652	42	125	61	GL50803 28651	0	2	0	GL50803 6262	8	11	9
GL50803 98054	10	57	1	GL50803 1770	0	2	0	GL50803 6283	0	8	0
GL50803 16636	1	14	0	GL50803 41451	0	2	0	GL50803 6289	49	16	28
GL50803 16924	0	7	0	GL50803 5070	0	2	0	GL50803 6304	0	14	0
GL50803 1345	31	129	31	GL50803 12095	0	2	0	GL50803 6330	12	14	14
GL50803 9810	7	33	10	GL50803 21943	0	2	0	GL50803 6372	0	12	0
GL50803 16717	5	6	2	GL50803 30476	0	2	0	GL50803 6430	102	83	129
GL50803 93011	0	6	0	GL50803 16766	0	2	0	GL50803 6464	0	39	7
GL50803 16376	1	16	0	GL50803 3896	0	5	0	GL50803 6469	0	4	0
GL50803 16124	20	101	2	GL50803 113038	0	2	0	GL50803 6564	0	7	0
GL50803 14622	0	37	2	GL50803 7566	0	2	0	GL50803 6617	4	12	0
GL50803 17411	4	63	2	GL50803 17229	0	2	0	GL50803 6633	0	16	14
GL50803 12885	6	36	12	GL50803 23492	0	2	0	GL50803 6687	122	146	245
GL50803 101278	5	50	5	GL50803 14277	0	2	0	GL50803 6709	8	2	2
GL50803 17482	5	50	0	GL50803 114192	0	2	0	GL50803 6744	0	4	0
GL50803 101906	1	10	0	GL50803 16653	0	2	0	GL50803 6752	0	3	0
GL50803 7350	0	5	2	GL50803 13771	0	7	0	GL50803 6835	0	0	9
GL50803 37350	4	5	1	GL50803 28696	0	2	0	GL50803 6939	8	4	0
GL50803 12102	14	73	8	GL50803 103164	0	2	0	GL50803 7007	16	3	14
GL50803 9030	16	63	15	GL50803 8917	0	7	0	GL50803 7009	0	3	2
GL50803 15567	2	29	0	GL50803 16731	0	7	0	GL50803 7030	0	4	7
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GL50803 16993	0	9	1	GL50803 11380	0	5	0	GL50803 7082	20	12	2
GL50803 2101	16	21	11	GL50803 5665	0	2	0	GL50803 7110	135	54	71
GL50803 112304	113	444	153	GL50803 6563	0	5	0	GL50803 7130	0	6	0
GL50803 2980	10	32	16	GL50803 3419	0	5	0	GL50803 7188	12	64	9
GL50803 9134	5	4	6	GL50803 4954	0	2	0	GL50803 7195	0	39	14
GL50803 10181	0	4	1	GL50803 9508	0	2	0	GL50803 7204	33	34	40

GL50803 8002	0	4	1	GL50803 112518	0	9	0	GL50803 7207	0	21	0
GL50803 8235	0	4	0	GL50803 10570	0	7	0	GL50803 7233	4	6	0
GL50803 17547	1	108	33	GL50803 10341	0	5	0	GL50803 7242	12	6	0
GL50803 93358	38	156	12	GL50803 7306	0	5	0	GL50803 7244	69	28	7
GL50803 100906	1	11	1	GL50803 9894	0	2	0	GL50803 7270	0	2	0
GL50803 14413	0	7	5	GL50803 14584	0	2	0	GL50803 7287	0	3	14
GL50803 16588	34	145	131	GL50803 16596	0	2	0	GL50803 7309	8	17	2
GL50803 9558	1	13	0	GL50803 6835	0	2	0	GL50803 7323	16	5	9
GL50803 17090	51	80	39	GL50803 6404	0	5	0	GL50803 7350	0	7	0
GL50803 15869	10	64	17	GL50803 9703	0	2	0	GL50803 7439	45	40	64
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Table S2.5. Primer sequences and workflow for tagging each protein

Gene	Plasmid	Primer Sequences	Episomal or Integrated	Notes
GL50803_4239	pks_4239_mNeonGreen-NEO	F- 5' ctccaccgcggtggcgccgcGCGCCCTCCCA CGCAACT 3' R-5' cctgaaccgcctccggatccCATCCACTCGAGC TTGACCATCG 3'	Integrated	Construct with C-terminal tag, 4239 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. BstBI was used for linearization.
GL50803_4259	pks_4259_mNeonGreen-NEO	F-5' tggagctccaccgcggtggcgccgcAGCGTCAT GGCTTCCCGC 3' R-5' ctcgcctgaaccgcctccggatccTTTGTGAGG AGCTCGTTGGC 3'	Episomal	Construct with C-terminal tag, 4259 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly.
GL50803_4383	pks_4383_mNeonGreen-Neo	F- 5' ctccaccgcggtggcgccgcTTATGACGTGT ATTCTTGTG 3' R-5' cctgaaccgcctccggatccATCCTGAAGA GATCTCAAGTAG 3'	Integrated	Construct with C-terminal tag, 4383 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. Aval was used for linearization.
GL50803_4463	pks_4463_mNeonGreen-Neo	F- 5' tggagctccaccgcggtggcgccgcGTAGACTA TATAATAACTGCTTTAGTAGGCCAC 3' R-5' ctcgcctgaaccgcctccggatccTTCCCGGTCA TCGTCAGTG 3'	Integrated	Construct with C-terminal tag, 4463 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector ; Gibson reaction was used for assembly. NheI was used for linearization.
GL50803_5328	pks_5328_mNeonGreen-Neo	F- 5' tggagctccaccgcggtggcgccgcCCCGTGGA CTCCTTTTGTG 3' R-5' ctcgcctgaaccgcctccggatccAAACTTATC GATCTGGGAGAC 3'	Integrated	Construct with C-terminal tag, 5328 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. BstBI was used for linearization.
GL50803_5800	pks_5800_mNeonGreen-Neo	F- 5' ctccaccgcggtggcgccgcCTACAGGTCTT AGTTAGTGCATAG 3' R-5' cctgaaccgcctccggatccCTTAAGGAGCTTC TCTAGAGTCG 3'	Integrated	Construct with C-terminal tag, 5800 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. XhoI was used for linearization.
GL50803_5810	pks_5810_mNeonGreen-Neo	F- 5' ctccaccgcggtggcgccgcTGCCGTATATC ATTGTAGTACCG 3' R-5' cctgaaccgcctccggatccATTCTTGATGCGC CAGGC 3'	Episomal	Construct with C-terminal tag, 5810 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly.
GL50803_6242	pks_6242_mNeonGreen-Neo	F- 5' ctccaccgcggtggcgccgcAAATTCCGGAT TTTACAGAAAAC 3' R-5' cctgaaccgcctccggatccTCAGACCTTCTCG GACTTG 3'	Episomal	Construct with C-terminal tag, 6242 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly.

GL50803_7323	pks_PPR_mNeon_7323_PAC	Promoter F-5' cggccgctctagaactagtggatccGCTCAAGAG AAGATCTTTAGAAATAC 3' Promoter R-5' tgctcaccaTTTTTTCGATTATACTAAAT TAACTTTTTTG 3' mNeonGreen tag F-5' atcgaaaaaATGGTGAGCAAGGGCGAG GAG 3' mNeonGreen tag R-5' ccgctcccatGGATCCGCTTCCGCCGGA 3' Coding Sequence F-5' aagcgatccATGGGAGCGGAACCTCTCA C 3' Coding Sequence R-5' gatgcaaaggctcgacggaagaattcCTACTGTGC CTCATATTCGTTC 3'	Episomal	Construct with an N-terminal tag, 200 base pairs of upstream sequence of 7323 were amplified as the putative promoter region from WBC6 genomic DNA. Sequences of the mNeonGreen tag was amplified from the mNeonGreen C-18 plasmid were also amplified as well as the coding sequence of 7323. The pks mNeonGreen N11 PAC vector was digested with BamHI-HF and EcoRI-HF restriction enzymes to remove the tag . All four fragments were assembled using In-Fusion cloning reaction (Takara Bio).
GL50803_8044	pks_8044_mNeonGreen_Neo	F-5' ccaccgcggtggcgccgctctagaATGCCCTGC TACTGTG 3' R-5' ccaccgcggtggcgccgctctagaATGCCCTGC TACTGTG 3'	Integrated	Construct with C-terminal tag, 8044 coding sequence was amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. HindIII-HF was used for linearization.
GL50803_8560	pks_8560_mNeon_Neo	F-5' ctccaccgcggtggcgccgcCAACGGTGTCT GCAGGCAC 3' R-5' cctgaaccgcctccggtatccGTACGCTGTCATG AATGGATTG 3'	Integrated	Construct with C-terminal tag, 8560 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. AgeI was used for linearization.
GL50803_8726	pks_8726_mNeon_Neo	F-5' tggagctccaccgcggtggcgccgcCAGTTAATT CAAACCGCTG 3' R-5' ctcgcctgaaccgcctccggtatccAACCTCAAA GGCAACTGG 3'	Episomal	Construct with C-terminal tag, 8726 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes ScaI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly.
GL50803_8854	pks_8854_mNeon_Neo	F-5' tggagctccaccgcggtggcgccgcGCCGAATCC ATTGCAGAG 3' R-5' ctcgcctgaaccgcctccggtatccTAAATCTGC AGTTGTGAGCAG 3'	Episomal	Construct with C-terminal tag, 8854 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes ScaI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly.
GL50803_9030	pks_9030_mNeon_Neo	F-5' ctccaccgcggtggcgccgcTTGCCAAGAAT TAATTGC 3' R-5' cctgaaccgcctccggtatccAACATTCTTAGAG TCGGCC 3'	Episomal	Construct with C-terminal tag, 9030 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly.
GL50803_9848	pks_9848_mNeon_Neo	F-5' ctccaccgcggtggcgccgcAGTGCAAGACA TCAGCAAAC 3' R-5' cctgaaccgcctccggtatccACCAGACTTGAA GATCAGG 3'	Integrated	Construct with C-terminal tag, 9848 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes ScaI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. Aval was used for linearization

GL50803_9861	pks_9861_mNeon_Neo	F– 5' ctccaccgcggtggcgccgcTGTATGCACA GAGCTGG 3' R–5' cctgaaccgcctccggatccGTTACATCCGAC ACCGTAG 3'	Episomal	Construct with C-terminal tag, 9861 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly.
GL50803_10038	pks_10038_mNeon_Neo	F– 5' ctccaccgcggtggcgccgcCAGCTAGTGGC CACCATTTTTTC 3' R–5' cctgaaccgcctccggatccTATCTTCCAGACT GTTGCAAGTG 3'	Episomal	Construct with C-terminal tag, 10038 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly.
GL50803_10429	pks_10429_mNeon_Neo	F– 5' ctccaccgcggtggcgccgcTCGGAATGAA CACCTGG 3' R–5' cctgaaccgcctccggatccCTCCTCTGCCTTCT CTTCG 3'	Episomal	Construct with C-terminal tag, 10429 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly.
GL50803_11354	pks_11354_mNeon_Neo	F– 5' ctccaccgcggtggcgccGCCTTGCTGGCA TAAGATC 3' R–5' cctgaaccgcctccggatccCTTAGCTCTCATG GCGTC 3'	Episomal	Construct with C-terminal tag, 11354 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly.
GL50803_11684	pks_11684_mNeon_Neo	F– 5' tggagctccaccgcggtggcgccgcTCCTTTCC GGCTGGGAAG 3' R–5' ctccgcctgaaccgcctccggatccGGCATAGTC GTGTCCAGTCAAT 3'	Integrated	Construct with C-terminal tag, 1935 base pairs of 11684 coding sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. NheI was used for linearization
GL50803_12999	pks_12999_mNeon_Neo	F– 5' tggagctccaccgcggtggcgccgcGAATAAGC AGAACGACACAAC 3' R–5' GCTGATGCCAAGACAGACgcatccggag gcggttcaggcggag 3'	Episomal	Construct with C-terminal tag, 12999 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly.
GL50803_13774	pks_13774_mNeon_Neo	F– 5' tggagctccaccgcggtggcgccgcGTTCTGGGG GAGTTGTGGAG 3' R–5' CGATGATACATGGGCCTTATACCgcat ccggaggcgggtcaggcggag 3'	Episomal	Construct with C-terminal tag, 13774 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly.
GL50803_13930	pks_13930_mNeon_Neo	F– 5' ctccaccgcggtggcgccgcGACGCGGTGGC CGGGGGG 3' R–5' cctgaaccgcctccggatccGCTAGTCTCCTTC CAGCGCCGCTCAATAG 3'	Integrated	Construct with C-terminal tag, 13930 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. MluI was used for linearization
GL50803_14213	pks_14213_mNeon_Neo	F– 5' tggagctccaccgcggtggcgccgcTCATCTCG CTCTTCGTCG 3'	Integrated	Construct with C-terminal tag, 14213 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-

		R-5' ctccgcctgaaccgcctccggatccCTTGGTGTGC TCCTCTGC 3'		HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. BstBI was used for linearization
GL50903_14299	pks_14299_mNeon_Neo	F-5' ctttctgtgactggtagtactCCAGACTTTCAA TTTAAGTGGATATGGGG 3' R-5' ctccgcctgaaccgcctccggatCCCTTCTGCGC CGCAACC 3'	Episomal	Construct with C-terminal tag, 14299 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes ScaI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly.
GL50803_14551	pks_14551_mNeon_Neo	F-5' ctccaccgcggtggcgccgcTACAGGGCGTA TGGGTCC3' R-5' cctgaaccgcctccggatccCATAACACGCCA GAGGATTTTAATC 3'	Integrated	Construct with C-terminal tag, 14551 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. ClaI was used for linearization
GL50803_15097	pks_15097_mNeon_Neo	F-5' ctccaccgcggtggcgccgcTGAGAGGACAG GGCGGTC 3' R-5' cctgaaccgcctccggatccCAACTCGAGAGT CATCGGC 3'	Integrated	Construct with C-terminal tag, 15097 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. AccI was used for linearization
GL50803_15124	pks_15124_mNeon_Neo	F-5' tggagctccaccgcggtggcgccgcGAATACCG CCTGACGACTC 3' R-5' ctacgtcattCATAGCGTTTTAGACTTTTCG C 3' F-5' aaacgctatgAATGACGTAGAGGCTACAA TAAAGAGAATTAG 3' R-5' ctccgcctgaaccgcctccggatccGGGCTTGAA CTCGGCGTC 3'	Episomal	Two sets of primers were used to remove an intron, totaling the entire coding sequence of 15124 plus 200 base pairs upstream for the putative promoter region. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; All three fragments were assembled using In-Fusion cloning reaction (Takara Bio).
GL50803_15591	pks_15591_mNeon_Neo	F-5' tggagctccaccgcggtggcgccgcCTTGTCGC TCAACGGAAG 3' R-5' ctccgcctgaaccgcctccggatccCAAGTATGT GAAAGCAGCATC 3'	Integrated	Construct with C-terminal tag, 1950 base pairs of 15591 coding sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. PpuMI was used for linearization
GL50803_16522	pks_16522_mNeon_Neo	F-5' tggagctccaccgcggtggcgccgcGTATGCGC ACGACGAGGTAC 3' R-5' ctccgcctgaaccgcctccggatccTCTGTAATAG TCTATCCACTGGACAC 3'	Integrated	Construct with C-terminal tag, 16522 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. PfoI was used for linearization
GL50803_16844	pks_16844_mNeon_Neo	F-5' ctccaccgcggtggcgccgcCTACAGATCCC GTAAACC 3' R-5' cctgaaccgcctccggatccATAACAGAGAC GTTTGCTAAC 3'	Integrated	Construct with C-terminal tag, 16844 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. Aval was used for linearization

GL50803_17153	pks_17153_mNeon_Neo	F- 5' ctccaccgcggtggcgccgcATACGTGTTAC AGCCTTCTG 3' R-5' cctgaaccgcctccggatccTTAGCAAGCTTC CAGAAC 3'	Integrated	Construct with C-terminal tag, 17153 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. PfoI was used for linearization
GL50803_17230	pks_17230_mNeon_Neo	F- 5' ctccaccgcggtggcgccgcGCAGCAGAAGC TCAGGAC 3' R-5' cctgaaccgcctccggatccACAACATTGCATC AGGAG 3'	Integrated	Construct with C-terminal tag, 17230 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. NheI was used for linearization
GL50803_17255	pks_17255_mNeon_Neo	F- 5' ctccaccgcggtggcgccgcGATACAATAGC ACATCCTCAAAG 3' R-5' cctgaaccgcctccggatccATGGAAATTCAA GAACTCC 3'	Integrated	Construct with C-terminal tag, 17255 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. NheI was used for linearization
GL50803_22855	pks_22855_mNeon_Neo	F- 5' tggagctccaccgcggtggcgccgcGTAATTCA CAGATCTTAAAGAATGTCTCC 3' R-5' ctccgcctgaaccgcctccggatccCTTGGACTG GGACTGCGC 3'	Integrated	Construct with C-terminal tag, 22855 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. Aval was used for linearization
GL50803_24451	pks_24451_mNeon_Neo	F- 5' tggagctccaccgcggtggcgccgcCGGCGGGTT CATAGTATATTC 3' R-5' ctccgcctgaaccgcctccggatccGTAGTTGGTC GGCCATGAC 3'	Episomal	Construct with C-terminal tag, 24451 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes ScaI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly.
GL50803_27925	pks_27925_mNeon_Neo	F- 5' tggagctccaccgcggtggcgccgcTGCTGAAG AGGACGTCCC 3' R-5' ctccgcctgaaccgcctccggatccGTTGAAAAG GGAGAGGAG 3'	Episomal	Construct with a C-terminal tag, 200 base pairs of upstream sequence of 27925 were amplified as the putative promoter region from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly.
GL50803_33685	pks_33685_mNeon_Neo	F- 5' ctccaccgcggtggcgccgcGCTGTACGAAA AGCTGGTG 3' R-5' cctgaaccgcctccggatccCATATGCTTTTTT GCCGTAAG 3'	Integrated	Construct with C-terminal tag, 1992 base pairs of 33685 coding sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. HindIII was used for linearization
GL50803_33989	pks_PPR_mNeonGreen_33989_PAC	Promoter F-5' tggagctccaccgcggtggcgccgcCTCTGGGT CTTCCATGTC 3' Promoter R-5' tgcctaccatTTTAAATAGAAAATGCCTCC 3' mNeonGreen F-5' ttctattaaaaATGGTGAGCAAGGGCGAGG AG 3' mNeonGreen R-5' ttctattaaaaATGGTGAGCAAGGGCGAGG AG 3'	Episomal	Construct with an N-terminal tag, 200 base pairs of upstream sequence of 33989 were amplified as the putative promoter region as well as the coding sequence of 33989 from WBC6 genomic DNA. Sequences of the mNeonGreen tag from the mNeonGreen C-18 plasmid were also amplified. The pks mNeonGreen N-11 PAC vector was digested with BamHI-HF and EcoRI-HF restriction enzymes to remove the tag. All four fragments were assembled using In-Fusion cloning reaction (Takara Bio).

		Coding sequence F-5' aagcggagcATGCAAAGTTGGCCATTT G 3' Coding sequence R-5' gatgcaaaggtcgacggaagTCAATGTTACG AAGCTCC 3'		
GL50803_21423	pka_21423_mNeon_NEO	F-5' tggagctccaccgcggtggcgccgcGTTTCGTCA ACGCGCTTCC 3' R-5' ctccgctgaaccgctccggatccCTTCAGTATG GAAAACTTGAGGC 3'	Integrated	Construct with C-terminal tag, 1665 base pairs of 21423 coding sequence were amplified from WBC6 genomic DNA. Restriction enzymes Not-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. PpuMI was used for linearization
GL50803_101212	pks_101212_mNeon_Neo	F-5' tggagctccaccgcggtggcgccgcGCGAGACG AGAATACCTAGAAAAGC 3' R-5' ctccgctgaaccgctccggatccGAGCGAGCA CCTGGCTGC 3'	Integrated	Construct with C-terminal tag, 1839 base pairs of 101212 coding sequence were amplified from WBC6 genomic DNA. Restriction enzymes Not-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. ClaI was used for linearization
GL50803_102813	pks_102813_mNeon_Neo	F-5' ctccaccgcggtggcgccgcTATCCTAGGTG CGTTGAG 3' R-5' cctgaaccgctccggatccTTCGAGAGGAT ATCTACTATCTC 3'	Integrated	Construct with C-terminal tag, 498 base pairs of 102813 coding sequence were amplified from WBC6 genomic DNA. Restriction enzymes Not-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. AgeI was used for linearization
GL50803_103855	pks_103855_mNeon_Neo	F-5' ctccaccgcggtggcgccgcATGTCTCAGCA GTTCATTCTC 3' R-5' cctgaaccgctccggatccAGAATCTTCAAA CTTCTTAAGATATG 3'	Integrated	Construct with C-terminal tag, 1361 base pairs of 103855 coding sequence were amplified from WBC6 genomic DNA. Restriction enzymes Not-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. XmaI was used for linearization
GL50803_113622	pks_113622_mNeon_Neo	F-5' ctccaccgcggtggcgccgcGCAGCAGAAGC TCAGGAC 3' R-5' cctgaaccgctccggatccACAACATTGCATC AGGAG 3'	Integrated	Construct with C-terminal tag, 2472 base pairs of 113622 coding sequence were amplified from WBC6 genomic DNA. Restriction enzymes Not-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. PpuMI was used for linearization
GL50803_115478	pks_115478_mNeon_Neo	F-5' ctccaccgcggtggcgccgcGACGAGACGCG CCGCGAG 3' R-5' cctgaaccgctccggatccCAGGTAGCTGAA CCTCGCCGG 3'	Integrated	Construct with C-terminal tag, 498 base pairs of 115478 coding sequence were amplified from WBC6 genomic DNA. Restriction enzymes Not-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. XhoI was used for linearization
GL50803_23833	pks_PPR_3H_A_23833_PAC	F-5' cgaattggagctccaccgcggtggcgccgcCGACT CTCCACCGCCCA 3' R-5' ccttgcaccatTTTATTTTGAAGGTTT TTACTCTTGTTCAGAAAC 3' F-5' tttaaaaataaaATGGTGAGCAAGGGCGA GGAG 3' R-5' tctggaagccatGGATCCGCTTCCGCCGG A 3'	Episomal	Construct with an N-terminal tag, 200 base pairs of upstream sequence of 23833 were amplified as the putative promoter region as well as the coding sequence of 23833 from WBC6 genomic DNA. Sequences of the mNeonGreen tag from the mNeonGreen C-18 plasmid were also amplified. The pks mNeonGreen N-11 PAC vector was digested with NotI-HF and EcoRI-HF restriction enzymes to remove the tag. All four fragments were assembled using In-Fusion cloning reaction (Takara Bio).

		F-5' cggaagcggatccATGGCTTTCCAGACCAA AG 3' R-5' acgatgatgcaaaggtcgacggaagTCAGTTTCC GAGGAAGATC 3'		
GL50803_8854	pks_8854_3 HA_Neo	F-5' gcaggaattcgatatcaagcttGCCGAATCCATT GCAGAG 3' R-5' cgtcataaggatattcctaagTAAATCTGCAGT TGTGAGCAG 3'	Integrated	Construct with C-terminal tag, 8854 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes AflIII and HindIII-HF were used to digest pks 3HA NEO vector; Gibson reaction was used for assembly. XcmI was used for linearization.
GL50803_12999	pks_12999_3 HA_Neo	F-5' gcaggaattcgatatcaagcttGAATAAGCAGA ACGACACAAC 3' R-5' cgtcataaggatattcctaagGTCTGTCTTGGC ATCAGC 3'	Integrated	Construct with C-terminal tag, 12999 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes AflIII and HindIII-HF were used to digest pks 3HA NEO vector; Gibson reaction was used for assembly. PfoI was used for linearization.
GL50803_13774	pks_13774_3 HA_Neo	F-5' gcaggaattcgatatcaagcttGTTCTGGGGGAGT TGTGGAG 3' R-5' cgtcataaggatattcctaagGGTATAAGGCC ATGTATCATCG 3'	Integrated	Construct with C-terminal tag, 13774 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes AflIII and HindIII-HF were used to digest pks 3HA NEO vector; Gibson reaction was used for assembly. AccI was used for linearization.
GL50803_27925	pks_27925_3 HA_Neo	F-5' gcaggaattcgatatcaagcttCAAGGTTGACTT GAACACTC 3' R-5' cgtcataaggatattcctaagGTTGAAAAGGGA GAGGAG 3'	Integrated	Construct with C-terminal tag, 1290 base pairs of 27925 coding sequence were amplified from WBC6 genomic DNA. Restriction enzymes AflIII and HindIII-HF were used to digest pks 3HA NEO vector; Gibson reaction was used for assembly. XcmI was used for linearization.
GL50803_103855	pks_103855_3 HA_Neo	F-5' gcaggaattcgatatcaagcttATGTCTCAGCAG TTCATTCTC 3' R-5' cgtcataaggatattcctaagAGAATCTTCAAA CTTCTTAAGATATG 3'	Integrated	Construct with C-terminal tag, 1361 base pairs of 103855 coding sequence were amplified from WBC6 genomic DNA. Restriction enzymes AflIII and HindIII-HF were used to digest pks 3HA NEO vector; Gibson reaction was used for assembly. PpumiI was used for linearization.
GL50803_33989	pks_PPR_3H A_33989_P AC	F-5' gcattttctattaaaaatgcttaagCTCTGGGTCTT CCATGTC 3' R-5' aaggatattcCATTTTAAATAGAAAATGC CTC 3' F-5' tattaaaaatgGAATATCCTTATGACGTCC C 3' R-5' aactttgcatTCAGGATCCAGCGTAATC 3' F-5' cgctggatccATGCAAAGTTGGCCATTTG 3' R-5' ctcctgtaattcagttgccaagcttTCAATGTTAC GAAGCTCC 3'	Episomal	Construct with an N-terminal tag, 200 base pairs of upstream sequence of 33989 were amplified as the putative promoter region as well as the coding sequence of 33989 from WBC6 genomic DNA. Sequences of the 3HA tag from the pks 3HA NEO plasmid were also amplified. The pks mNeonGreen N-11 PAC vector was digested with BamHI-HF and EcoRI-HF restriction enzymes to remove the tag. All four fragments were assembled using In-Fusion cloning reaction (Takara Bio).

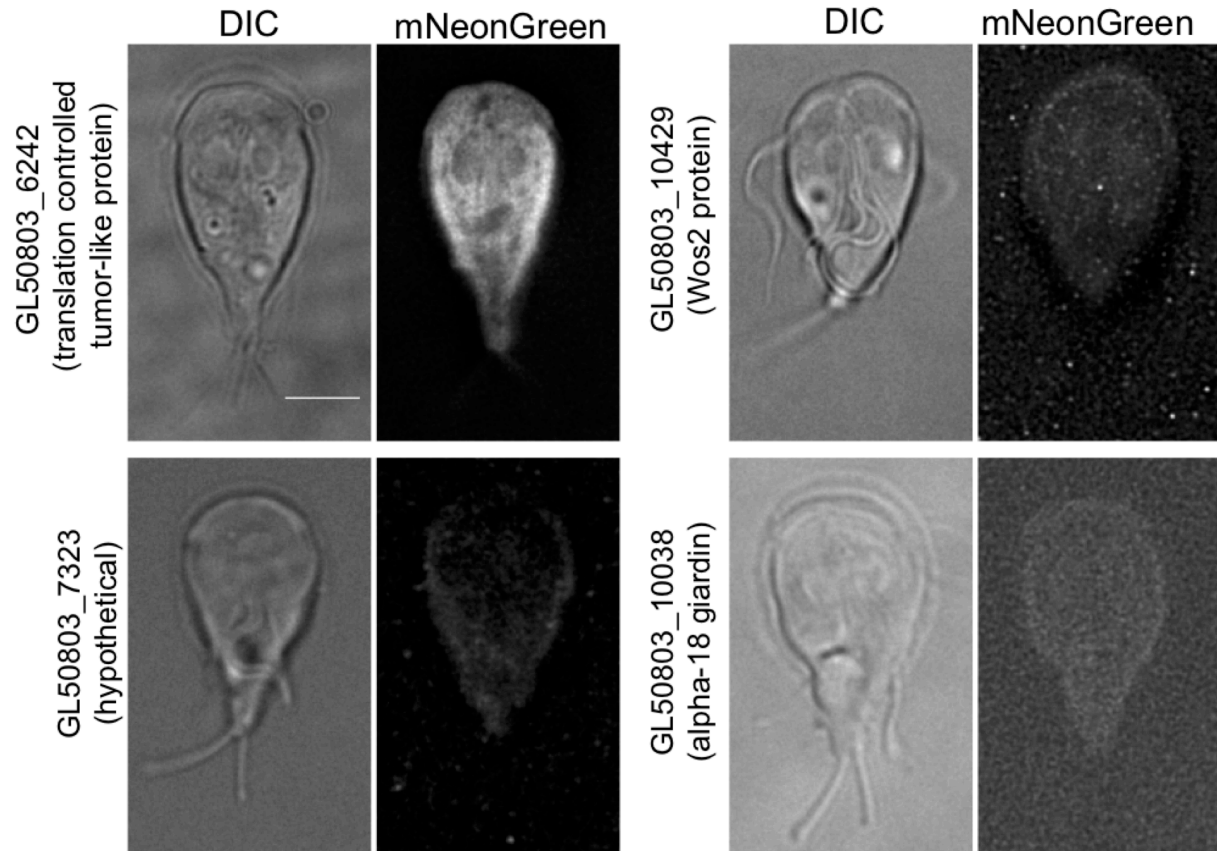


Figure S2.11. Putative GlActin interactors with non-specific localization or low signal

Five proteins had non-specific localization, including faint cytoplasmic localization in GL50803_7323, GL50803_10429, and GL50803_10038. GL50803_6242 and GL50803_16522 were distributed throughout the cytoplasm. Scale bar denotes 5 μ m.

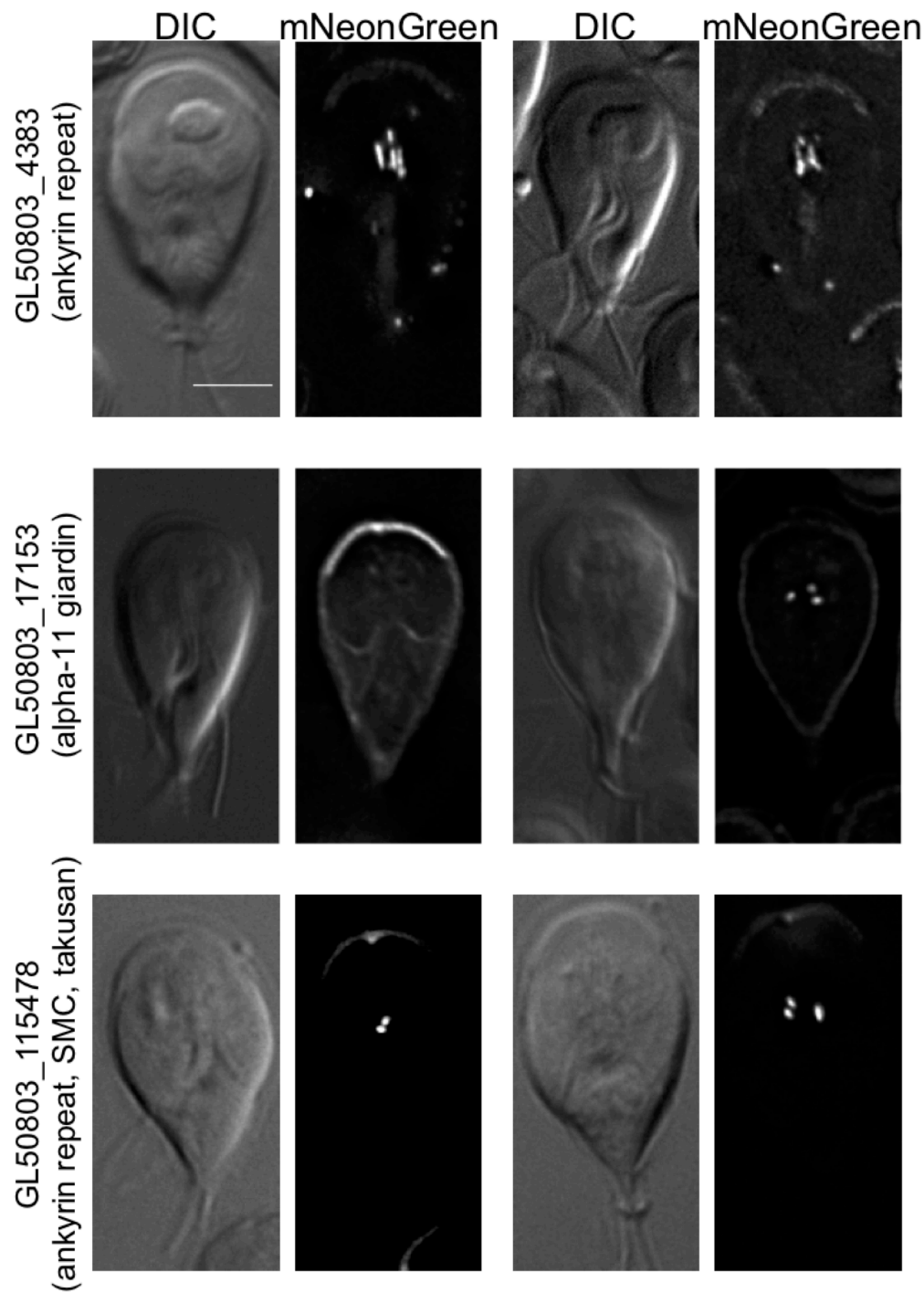


Figure S2.12. Range of mitosome-like localizations in marginal plate proteins

The marginal plate proteins GL50803_17153, GL50803_4383, and GL50803_115478 all localized to axoneme-associated structures which vary in number.

2.2 CHAPTER 2 REFERENCES

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Chapter 3. DISC AND ACTIN-ASSOCIATED PROTEIN 1

INFLUENCE HOST ATTACHMENT IN THE INTESTINAL

PARASITE *GIARDIA LAMBLIA*

Abstract

The deep-branching eukaryote *Giardia lamblia* is an extracellular parasite that attaches to the host intestine via a microtubule-based structure called the ventral disc. Control of attachment is mediated in part by the movement of two regions of the ventral disc that either permit or exclude the passage of fluid under the disc. Several known disc-associated proteins (DAPs) contribute to disc structure and function, but no force-generating protein has been identified among them. We recently identified several *Giardia* actin (*GI*Actin) interacting proteins at the ventral disc, which could potentially employ actin polymerization for force generation and disc conformational changes. One of these proteins, Disc and Actin Associated Protein 1 (DAAP1), is highly enriched at the two regions of the disc previously shown to be important for fluid flow during attachment. In this study, we investigate the role of both *GI*Actin and DAAP1 in ventral disc morphology and function. We confirmed interaction between *GI*Actin and DAAP1 through coimmunoprecipitation, and used immunofluorescence to localize both proteins throughout the cell cycle and during trophozoite attachment. Similar to other DAPs, the association of DAAP1 with the disc is stable, except during cell division when the disc disassembles. Depletion of *GI*Actin by translation-blocking antisense morpholinos resulted in both impaired attachment and defects in the ventral disc, indicating that *GI*Actin contributes to disc-mediated attachment. Depletion of DAAP1 through CRISPR interference resulted in intact discs but impaired attachment, gating, and flow under the disc. As attachment is essential for infection, elucidation

of these and other molecular mediators is a promising area for development of new therapeutics against a ubiquitous parasite.

Author Summary

Giardia lamblia is a single-celled organism and one of the most common gastrointestinal parasites worldwide. In developing countries, recurrent *Giardia* infections are common, due to lack of access to clean water. *Giardia* infections can lead to diarrhea, vomiting, dehydration, disruption of the intestinal microbiome, and chronic infections can lead to colitis and irritable bowel syndrome. Because existing drug treatments have side effects and *Giardia*'s resistance to drugs is increasing, new treatment strategies are needed. The parasite's attachment to the host's intestine is mediated by a *Giardia*-specific structure that resembles a suction cup and is called the ventral adhesive disc. We previously identified DAAP1, a protein which interacts with *Giardia* actin and localizes to the ventral disc. Here, we explore the relationship between these two proteins and investigate their role in disc-based attachment. Most disc proteins, including DAAP1, are unrelated to any human proteins, making them appealing drug targets to inhibit parasite attachment and infection.

Introduction

Giardia lamblia, a single-celled parasite, is the cause of over 280 million annual cases of the gastrointestinal disease giardiasis worldwide [1,2] This extracellular parasite colonizes the lumen of the intestine, where its ability to maintain infection requires tight attachment to microvilli, in order to withstand the peristaltic flow of the gastrointestinal tract [3]. The primary means of attachment is via a specialized microtubule-based organelle known as the ventral

adhesive disc [4–6]. A well-functioning disc is critical for cell survival and disease transmission [7], but the molecular mechanisms controlling conformational dynamics and function remain poorly understood.

Imaging of fluid flow at the disc margin identified two areas postulated to be important for tight attachment: one at the anterior and the other at the posterior of the disc [8,9] (Figure 3.1.

Depletion of *GLActin* affects attachment and ventral disc morphology). The former is the marginal region of the overlap zone, the region of the disc where the sheet of microtubules overlaps itself. The other is the ventral groove, a hump in the posterior region of the disc where the ventral flagella exit between the disc and cell body. Interruption of contact in these areas results in disruption of attachment and in fluid moving underneath the disc [9–11]. Cryo-electron tomography imaging of the disc reveals different protein densities in these areas, indicating specialized architecture within the disc itself, which may indicate that specific effector proteins control disc conformation and fluid flow [12]. In the current model, both hydrodynamic flow and suction contribute to the mechanism of attachment [9]. Attachment occurs in several distinct stages [8,9]. In early attachment, the anterior ventrolateral flange, lateral crest and ventral groove make contact with the surface while the ventral flagella beat. During this stage cells can either skim the surface or remain stationary. In the later stages of attachment, the cell body becomes more intimately associated with the surface so that the lateral shield and bare area make contact with the surface and the cells remain in place. Flagella-beating is required for initiation, but not maintenance, of attachment, indicating that fluid must be moved out from underneath the disc during the earlier stages of attachment [9,13].

These conformational changes in the disc which control attachment are not generated by microtubule turnover, as microtubule depolymerizing or stabilizing drugs do not affect

attachment [14]. Though microtubules are the foundation of the disc, it is also composed of over 100 other proteins, known as disc-associated proteins (DAPs). At least five of these DAPs have been shown to be important for disc biogenesis and function [9,11]. Only three DAPs identified to date contain known microtubule-binding motifs; most are ankyrin-repeat proteins, NEK kinases, or the annexin-like alpha-Giardins [14,15]. At least 25, however, lack identifiable domains and are annotated in the *Giardia* genome as “hypothetical proteins” [16,17]. So far, no DAPs have been identified as motor or force-generating proteins. Thus, the proteins that mediate changes in disc conformation are unknown

Actin has a conserved role in force generation and in controlling cell and organelle shape in eukaryotic cells[18]. Previously, localization studies using heterologous antibodies demonstrated that *Giardia* actin (*GlActin*) and actin regulators associate with the ventral disc [6,19]. This observation led to multiple experiments attempting to test the role of *GlActin* in attachment using actin inhibitors. These studies yielded conflicting results because reagents that typically recognize actin or actin-binding proteins in other organisms were presumed to act similarly in *Giardia* [6]. Later, *GlActin* was determined to be one of the most divergent actins found among eukaryotes and its genome lacked genes encoding conventional actin-binding proteins that were reportedly localized to the disc [20–22]. Further research revealed that *GlActin* lacks the binding sites for the drugs and anti-*GlActin* antibodies revealed localization throughout the cell [21]. Thus, *GlActin*’s potential role in disc-based attachment was dismissed as an artifact [21].

We recently identified eight *Giardia* actin (*GlActin*) interactors that localize to the ventral disc [17], suggesting a means to resolve the role of actin and actin-binding proteins in ventral disc function. One such actin-interacting protein, GL50803_16844, which we denote

Disc and Actin Associated Protein 1 (DAAP1), appears highly enriched in the two regions of the disc important for fluid flow during attachment.

In this study, we use morpholinos and CRISPR interference (CRISPRi) to deplete *GlActin* and DAAP1 respectively and investigate the role of both proteins in the ventral disc. Our results show that *GlActin* plays a role in normal ventral disc morphology and both *GlActin* and DAAP1 are required for parasite attachment.

Results

***GlActin* affects attachment and disc morphology**

In vitro, *Giardia* normally grows attached to the inside of a culture tube. We used an antisense translation-blocking morpholino to deplete *GlActin* for 24 hours, [21] after which we assessed their attachment. Here, we used an assay where *Giardia* cells are not challenged, comparing the numbers of cells attached to the tube to cells unattached in media in each condition. With an approximately 50% knockdown (Figure S3.8), we found attachment to plastic culture tube walls to be significantly decreased, with an average of 23% of cells attached compared to 75% of cells treated with a control morpholino (Figure 3.1B). Dead *Giardia* cells lose their ability to adhere to culture tubes. To account for the possibility that the majority of the unattached cells were dead, we stained the unattached cells with propidium iodide to mark dead cells, and fluorescein diacetate to mark live cells. While slightly more unattached *GlActin* knockdown cells were dead compared to that of the control population, this difference was not statistically significant (Figure 3.1C), indicating that the defect in attachment in *GlActin*-depleted cells was not due to a preponderance of dead cells.

We asked if the role of *GI*Actin in attachment could be attributed to an effect on the ventral disc. Attached and unattached cells were treated with either control or anti-*GI*Actin morpholino, fixed after 24 h, and examined for distribution of a ventral disc marker using immunofluorescence microscopy. We used a HALO-tagged delta-giardin which is widely distributed in the disc to monitor whether the disc appeared normal or defective. Defects were then further classified as 1) mild, with misshapen/broken discs or small gaps in the disc or 2) severe, including unwound discs or severely misshapen discs (Figure S3.9, Figure 3.1D, Figure 3.1E). More *GI*Actin knockdown cells had defective discs compared to those in the control; however, this difference was only significant in unattached cells (Figure 3.1D, Figure 3.1E). While both attached and unattached *GI*Actin-depleted cells had more disc defects than their counterparts treated with the control morpholino, the unattached *GI*Actin-depleted cells had the most defective discs among all the categories. Yet, there were more unattached cells than cells with disc defects, suggesting that *GI*Actin has a role in attachment beyond its contribution to disc morphology.

***GI*Actin interacts with disc-associated protein DAAP1**

We next asked if GL50803_16844, previously identified by mass spectrometry as a potential actin filament interactor at the ventral disc [17], could be involved in attachment. Hereafter, we will refer to this protein that lacks identifiable domains and homologues in other organisms as Disc- and Actin-Associated Protein 1 (DAAP1). We tagged DAAP1 with a C-terminal 3xHA tag and expressed it episomally in *Giardia*. Immunoprecipitation of this construct revealed it in complex with *GI*Actin (Figure 3.2A). We also endogenously tagged DAAP1 with a C-terminal mNeonGreen tag then used live and fixed cell fluorescent imaging. Notably, this

fusion protein localizes to the entirety of the disc, but it is highly enriched in the ventral groove and the overlap zone, two areas implicated in attachment (Figure 3.1A, Figure 3.2B). Flexion of the overlap zone can be seen during detachment from the surface, consistent with the notion that conformational changes of specific regions of the disc can regulate attachment (Supplemental Movie 1).

To investigate co-localization between *Gl*Actin and DAAP1, we calculated Pearson's correlation coefficients for these two proteins using the JACoP plugin for ImageJ [23,24]. While DAAP1-mNeonGreen does not appear to co-localize with *Gl*Actin during interphase, in mitosis DAAP1 leaves the ventral disc and was observed to co-localize with *Gl*Actin (Figure 3.2C).

We also measured the intensity of DAAP1-mNeonGreen fluorescence in *Gl*Actin-depleted cells, selecting a region in the ventral groove, as that is where DAAP1 expression is the highest (Figure 3.3). While there was no significant difference between fluorescence levels in attached cells (Figure 3.3A, Figure 3.3B), this difference was significant in unattached cells (Figure 3.3C, Figure 3.3D). Furthermore, in some *Gl*Actin-depleted cells, we observed DAAP1 loss of enrichment in the entire disc (Figure 3.3C). These results indicate that *Gl*Actin is responsible for DAAP1 disc localization.

The microtubules in the ventral disc are stable and do not undergo dynamic instability; they rely on a set of core DAPs to maintain this stability [14]. During mitosis, the disc is dismantled as the cells must detach to “swim” away from each other during cytokinesis [25,26]. New discs in the daughter cells appear during early cytokinesis and are built from microtubules originating from the mother disc and the median body [25,27]. Notably, DAAP1-mNeonGreen remains on the disc during telophase, late into disassembly, and begins to coalesce again on the daughter discs in mid cytokinesis. In newly-divided cells, DAAP1-mNeonGreen has returned to

its disc localization and is once more enriched in the ventral groove (Figure 3.3B). Fluorescence recovery after photobleaching (FRAP) confirmed the stability of DAAP1-mNeonGreen for disc localization, as fluorescence levels did not recover for 12 minutes after photobleaching (Figure 3.4) in either of the two regions of the disc where DAAP1 is enriched. Viability was confirmed by cell movement as seen in Supplemental Movie 2. This result is similar to the fluorescence recovery of other DAPs. Taken together, these data indicate that DAAP1 is a *Gl*Actin interactor and stably localized to the ventral disc in interphase.

DAAP1-depleted cells have intact ventral discs

Since *Gl*Actin is involved in both attachment and disc morphology, we asked if DAAP1 could have a similar role. We attempted depletion of DAAP1 with translation-blocking morpholinos, however, the sequence was design-resistant and the knockdown level inconsistent. We then turned to CRISPRi to investigate DAAP1's potential role in attachment and disc morphology. To monitor disc morphology as well as deplete DAAP1, we inserted the HALO-tagged delta-giardin marker into the dead Cas9- (dCas9) based CRISPRi plasmid and introduced this into a DAAP1-mNeonGreen expressing cell line. This allowed us to evaluate knockdown on both a whole population (Figure 3.5A) and an individual cell basis. We analyzed the fluorescence of endogenously-tagged DAAP1-mNeonGreen in control cells expressing dCas9 alone to those expressing a DAAP1 specific small guide RNA resulting in an average decrease in expression of 40% for the population. We once again separated cells that were attached to culture tubes from unattached cells to probe for disc defects in each subpopulation. We did observe a higher proportion of disc defects in the DAAP1-depleted cells than in the control, but this

difference was not significant (Figure 3.5B and Figure 3.5C). These results indicate that a 40% depletion of DAAP1 does not impact gross ventral disc morphology.

Inability to Maintain Attachment in DAAP1-Depleted Cells

DAAP1 knockdown cells had no visible defects, so DAAP1 may not be needed for disc assembly. To investigate a possible role in attachment, we used the non-challenge attachment assay on DAAP1-depleted cells and observed a small but significant decrease in attachment for two of three replicates relative to control cells (Table S3.7). As CRISPRi is known to have variable penetrance in *Giardia* [28], we analyzed the fluorescence in unattached and attached cells separately. Here we found reduced levels of DAAP1-mNeonGreen in unattached cells compared to attached ones (Figure 3.6A). This result prompted us to further explore the possible role of DAAP1 in attachment using a challenged attachment assay. In their normal intestinal habitat, *Giardia* must maintain attachment even when subjected to the flow of chyme. With this in mind, we challenged DAAP1-depleted cells with a fluid shear force. DAAP1-depleted and control cells were allowed to attach inside a flow chamber and then subjected to a flow of growth medium. Cell position and velocity were tracked using Trackmate (Figure 3.6B, [29]). DAAP1-depleted cells slid significantly faster under flow than their control counterparts (Figure 3.6C, Figure 3.6D). Furthermore, more control cells remained in the field of view than DAAP1-depleted cells over time (Figure 3.6E), indicating a better ability to maintain attachment.

Impaired Seal Formation in DAAP1-Depleted Cells

We then hypothesized that the impaired attachment in DAAP1-depleted cells may be due to impairments of the ventral groove or overlap zone “gates” that control fluid flow and buildup

of negative pressure differentials under the disc. We used fluorescent microspheres to investigate this fluid flow in live, attached cells imaged in normal growth media. We found that spheres entered under the disc more frequently in DAAP1-depleted than dCas9 control cells, indicating a defect in gating flow under the disc (**Error! Reference source not found.A**). Furthermore, we found more beads per cell in the DAAP1-depleted cells (Table S3.9).

Since the ability of the gates to close affects disc seal formation [8,13], we used plasma membrane stain, CellMask Orange and TIRF microscopy to confirm a defect in seal formation for DAAP1-depleted and dCas9-Control cells. This approach allowed us to assess the different levels of attachment based on which portions of the cell were in contact with the surface. Since stage 1 of attachment is unattached skimming, we omitted these cells from our analysis, and only included cells in stage 2 (lateral crest contact), stage 3 (lateral shield contact), and stage 4 (bare area contact). We found more cells at stages 2 and 3 and fewer in stage 4 in DAAP1-depleted cells than in dCas9-Control (**Error! Reference source not found.B**), indicating impaired seal formation around the ventral disc. Taken together, these data demonstrate that DAAP1 functions at the disc gates that regulate fluid flow and ventral disc seal formation.

Discussion

Here, we have confirmed that *GI*Actin has a role in ventral disc morphology and disc-based attachment. We also show that *GI*Actin interactor DAAP1 is a stable component of the ventral disc. While perturbation of DAAP1 levels was not associated with visible effects on disc morphology, it resulted in decreased attachment.

The ventral disc is well known as a *Giardia*-specific microtubule based structure [16,30] and our data show that the actin cytoskeleton has an important role in ventral disc assembly and

function. A functional relationship between actin and microtubules has been well-documented for other structures. In *Chlamydomonas*, actin is required for intraflagellar transport [31,32]. Actin monomers are a key component of the flagella through interaction with the inner dynein arm [33–36] and recent evidence indicates actin is a component of gamma-TURC [37]. Actomyosin interactions influence the orientation of the mitotic spindle [38,39] and F-actin associates directly with mitotic spindles in *Xenopus* and starfish embryos [40,41]. Actin is also involved in microtubule-based host interaction in other parasites. For instance, one of *Toxoplasma gondii*'s formins localize exclusively to the conoid [42], a latticelike microtubule organelle which initiates host invasion [43]. Triggered by release of calcium ions, actin polymerization, in coordination with myosins A and H, contributes to conoid extrusion to initiate host invasion [44–46]. *Gl*Actin polymerization could have an analogous role in controlling the movement of the ventral disc to facilitate attachment.

We also observed defects in the ventral disc in our *Gl*Actin-depleted cells, which we characterized as either mild (misshapen, broken, or with small gaps) or severe (unwound, severely misshapen, often associated with cytokinesis defects). There are several possible explanations for these phenotypes. During mitosis, the ventral disc is dismantled and two daughter discs are rebuilt during cytokinesis [25]. One possibility is that *Gl*Actin has a role in loading proteins on the assembling disc. *Gl*Actin also likely contributes to ventral disc positioning, since actin has a role in positioning basal bodies that nucleate the disc [47,48]. However, many of the cells with defective discs have normally-positioned flagella, indicating this defect is not entirely due to basal body mispositioning. While the resolution of light microscopy limits our ability to see details in the microtubule array of the disc [49], over 25% of

our unattached *GI*Actin-depleted cells lacked noticeable defects. Therefore, a portion of the attachment defect cannot be attributed to disc defects alone.

Another possibility is that *GI*Actin helps maintain the structure of the disc, consistent with its role in maintaining cell and organelle shape in other organisms [18,21]. The unusual stability of disc microtubules can be at least partially attributed to certain DAPs [8,14,16], which lock the microtubules into place, preventing their turnover. *GI*Actin interacts with at least one of these DAPs, GL50803_5188 [17], and could be working in conjunction with it to stabilize the disc microtubules themselves. Another explanation is that *GI*Actin works to stabilize one of the non-microtubule components of the ventral disc. The microtubules of the disc are overlaid by the giardin-composed microribbons [50], which are connected by crossbridges [12,51]. Since the spiral shape is often maintained, but unwound, in *GI*Actin-depleted cells, it may be that *GI*Actin contributes to establishing and/or maintaining disc overlap, as the overlap zone also functions as the anterior gate of the ventral disc.

A previous study from our lab identified eight *GI*Actin interactors which localize to the ventral disc, including DAAP1 [17]; here we confirmed their complex formation through co-immunoprecipitation. We did not detect co-localization between DAAP1 and *GI*Actin in interphase cells, but present evidence that suggests some co-localization during mitosis when new discs are assembled. Polymerized *GI*Actin may be enriched in the ventral disc at these stages, but detection may be obscured due to technical limitations. Unfortunately, use of the anti-*GI*Actin antibody does not differentiate between filaments and pools of monomeric *GI*Actin which likely obscures some filaments. Currently, there are no methods to specifically detect F-*GI*Actin, as Rhodamine-Phalloidin does not bind to *GI*Actin filaments [21]. Actin in *Toxoplasma gondii* was believed to be primarily monomeric and only capable of forming short filaments

[52,53]. However, expression of an anti-actin nanobody revealed a previously unseen complex network of F-actin [54]. Poor detection of *GI*Actin using the anti-*GI*Actin antibody could also be due to steric hindrance. Actin-binding proteins could sterically block access of anti-*GI*Actin antibodies to disc-localized *GI*Actin, similar to low anti-tubulin antibody staining compared to our ability to see microtubules of the disc with mNeonGreen-Tubulin [25]. Another possibility is that actin only transiently associates with the disc for specific conformational changes which would be challenging to capture in fixed cells. A live marker specific for *GI*Actin is needed to determine the extent of actin association with conformational dynamics of the ventral disc including disc doming, bare area protrusion and disc flexion as seen in Movie S1.

Similar to other disc-associated proteins [44], DAAP1's localization to the disc is stable, as we did not see any recovery of this protein for 12 minutes after photobleaching. While DAAP1 was previously identified as a potential disc interactor, it was not found in high enough abundance in the detergent-extracted disc proteome to qualify for further study as a disc-associated protein [14]. These results possibly indicating that this protein is a peripheral component of the disc, which would be expected in order for it to engage with a dynamic subset of *GI*Actin.

Using CRISPR-interference, we depleted DAAP1 from cells, and found a mild defect in attachment, but no significant impact on disc morphology. While a DAAP1 knockout would give us better insight into how DAAP1 loss affects the disc, the ability to readily knockout genes of interest remains under development. CRISPRi and Morpholino knockdown both have variable penetrance, and thus level of depletion fluctuates among cells, and we observed normal discs even in cells with very little DAAP1 fluorescence (Figure 3.5). In contrast, depletion of median body protein with morpholinos results in broken discs [11]. Although an inability to attach can

be attributed to disc defects [11,28], the depletion of gamma-giardin, another disc-localized protein that interacts with *GI*Actin [17], resulted in malformed discs with normal attachment [55]. These findings indicate that some disc proteins may be involved in maintaining the structural integrity of the disc, while others are involved in controlling conformational changes of the disc required for modulating attachment. Our data is consistent with DAAP1 being in the latter category. [12]

The possibility that some disc proteins contribute to attachment but not structure may also explain the disparity between the severity of attachment defects in *GI*Actin-depleted cells and DAAP1-depleted cells. Since many of the discs in *GI*Actin-depleted cells were unwound or misshapen, this suggests roles in building the disc or collaboration with DAPs that maintain the disc structure. However, since over 25% of our unattached *GI*Actin-depleted cells had apparently normal discs, the attachment defect cannot be attributed to disc defects alone. Our previous study identified eight other putative disc-localized F-*GI*Actin interactors which could also contribute to attachment and/or disc morphology [17].

An additional explanation for the severity of attachment defects in *GI*Actin-depleted cells is due to its involvements in supplemental mechanisms of attachment. *GI*Actin has been demonstrated to function in building the ventrolateral flange, *Giardia*'s membrane lamella which also contributes to attachment [56,57]. Further, the marginal plate, a rigid structure associated with the anterior axonemes, is also hypothesized to be involved in attachment [10,30]. *GI*Actin is stably enriched in this structure [21,58] and we have described six interactors which localize there as well [17]. Given *GI*Actin's involvement in all three structures hypothesized to augment attachment, depletion of *GI*Actin likely has multivariant effects on attachment, contributing to a more severe phenotype.

Clarity is needed on the nature of the DAAP1-actin interaction, both *in vitro* and *in vivo*. We have demonstrated that DAAP1 complexes with *Gl*Actin filaments but we cannot yet say if the interaction is direct. However, *Gl*Actin depletion results in decreased enrichment of DAAP1 at the ventral disc, further demonstrating a functional relationship between these proteins. DAAP1 could serve as an intermediary between *Gl*Actin and the disc, and the two could be working in conjunction to modulate disc conformation and attachment. Identification of other interactors of DAAP1 could also elucidate this interaction and potentially reveal more about the mechanism regulating disc conformational changes.

The biology of parasites is often unlike anything seen in other organisms, shaped by the unusual environments in which they live. *Giardia*'s ventral disc exemplifies parasitic innovation and makes this *Giardia* specific structure a target for drug development. Since most DAPs lack homologs, they are in theory potential drug targets; however, the lack of active sites reduces their potential [59]. Further understanding of the mechanisms of attachment and its regulation will increase the viability of developing drugs that could specifically function through regulating attachment. Actin, however, is a druggable protein and conventional actin drugs do not bind to *Gl*Actin or inhibit its function, rendering it an appealing therapeutic target.

Materials and Methods

Parasite Strain and Growth Conditions

G. lamblia strain WB clone 6 (ATCC 50803; American Type Culture Collection) was cultured as described previously [21].

Vector Construction

All constructs in this study were C-terminal fusions, made using Gibson reactions with linearized vectors and PCR products [60]. See Table S3.8 for primer sequences and workflow.

Live Cell Imaging

Cells were chilled with ice for 15 minutes to detach from the culture tube and then placed into an Attofluor cell chamber (Molecular Probes) and incubated in a GasPak EZ anaerobic pouch (BD) or a Tri-gas incubator (Panasonic) set to 2.5% O₂, 5% CO₂ for 90 minutes at 37°C. Cells were then washed four times with HEPES-buffered saline (HBS: 137mM NaCl, 5mM KCl, 0.91mM Na₂HPO₄-heptahydrate, 5.55mM Glucose, 20mM HEPES, pH7). Live cell imaging was performed on a DeltaVision Elite microscope (GE) equipped with DIC optics, using a 100 × 1.4 NA or 60 × 1.42 NA objective, and a sCMOS 5.4 PCIe air-cooled camera (PCO-TECH).

Coimmunoprecipitation

Cell cultures of 500 mL each of *Giardia* wildtype and DAAP1-3xHA were grown for 3 days, then iced for 2 hours to detach, and spun for 1500xg at 4°C for 20 minutes. Cells were then washed twice in 1X HBS with 2X HALT protease inhibitors and 10 μM chymostatin, 1 μM leupeptin, and 1 μM E64. Each pellet was resuspended to a final volume of 1.2 mL and 100 mM

DSP in DMSO was added to a final concentration of 1 mM and incubated at room temperature for 30 minutes. The reaction was quenched for 15 minutes with an addition of Tris pH 7.4 final concentration 20 mM. Cells were then pelleted by spinning for 7 minutes at 700xg and resuspended in 350 μ L lysis buffer (80 mM KCl, 10 mM imidazole, 1 mM MgCl₂, 1 mM EGTA, 5% Glycerol, 20 mM HEPES, 0.2 mM, CaCl₂, 10 mM ATP, 0.1% Triton X-100, 500 mM NaCl, pH 7.2, 2XHALT protease inhibitors and 10 μ M chymostatin, 1 μ M leupeptin, and 1 μ M E64). Cells were then lysed by sonication and cleared with a 10 minute spin at 10,000xg. A volume of 17.5 μ L of equilibrated EZview Red Anti-HA Affinity gel (Sigma) was added to each tube of lysate, then incubated at 4 °C with end-over-end mixing for 1 hour. Beads were then spun at 8,200xg for 30 seconds and the supernatant was discarded, followed by a total of three washes with 750 μ L wash buffer (80 mM KCl, 10 mM imidazole, 1 mM MgCl₂, 1 mM EGTA, 5% Glycerol, 20 mM HEPES, 0.2 mM CaCl₂, 10 mM ATP, 0.5% Tween, 500 mM NaCl, pH 7.2). Each wash consisted of end-over-end rotation for 5 minutes followed by a 30 second spin at 8,200 x g. Protein was then incubated with 50 μ L of 8 M Urea at RT for 20 minutes to elute. The beads were pelleted and then sample buffer was added to the supernatant, the sample was boiled for 5 mins at 98 °C, and run on a 12% SDS-PAGE gel, followed by western blot protocol described previously [21].

Immunofluorescence Microscopy

Fixation and imaging were performed as described in previous work [61], with the addition of a JaneliaFluor 646 HaloTag ligand (Promega, GA1120) or JaneliaFluor 549 HaloTag ligand (Promega, GA1110) at 1:100 in the second immunostaining step. Image analysis was performed in FIJI [62]. DAAP1 intensity was measured by generating a circular region of interest

surrounding the ventral groove and measuring fluorescence intensity at the brightest point in each image stack.

Morpholino knockdown

Morpholino knockdown was performed as described previously [63]. Cells were lysed and run on a 10% SDS-PAGE gel, followed by western blot protocol as described previously [17,21]. For each of three biological replicates, samples were divided equally into three lanes and intensity of each lane measured using FIJI and averaged. Cell counts were performed with a MoxiZ coulter counter to ensure the same number of cells were included in each sample. For morpholino sequences, see Table S3.8.

Assessment for Live/Dead Cells

Stock solutions of dye were created by dissolving 5 mg fluorescein diacetate (Sigma-Aldrich, F-7378) in 1 ml acetone and 2 mg propidium iodide (Sigma-Aldrich, P4170) in 1 ml HBS. A 2X stain solution was prepared by diluting the stock solutions of fluorescein diacetate and propidium iodide by 1:100 in 1X HBS. Cells were pelleted by centrifugation at 500 x g and resuspended in 1 mL 1X HBS. 1mL of 2x stain solution was then added to the cell suspension, washed twice in 1X HBS by centrifugation and resuspended in 1X HBS for imaging.

Attachment Assay

Cells were treated with either a standard control or anti-*G*/Actin morpholino and allowed to recover for 24 hours in a total volume of 13 mL of TYDK media at 37 °C. At 24 hours, each culture tube was inverted six times; detached cells in media were decanted and counted with a

MoxiZ coulter counter (Orflo), then replaced with 13 mL ice cold media. Culture tubes were placed on ice for 30 minutes to detach the remaining cells, after which these were also counted. Cells were then imaged live or fixed as described previously [25]. Three independent replicates of each experimental and control were analyzed.

Fluorescence Recovery after Photobleaching.

FRAP was performed on DAAP1-mNeonGreen in the ventral disc to measure protein turnover. Cells were chilled with ice for 20 minutes to detach from the culture tube and then placed into an Attofluor cell chamber (Molecular Probes) and incubated in a O₂/CO₂ incubator (Panasonic) for 1-2 hours at 37° C. Media was replaced with 37 °C 1X HBS before imaging. All experiments were conducted using a FRAP-enabled DeltaVision Spectris with 405 nm solid-state laser, 100% laser power, 50 mW laser, and 25 ms stationary pulse. Three images were taken until the bleach event for 0.5 second intervals, followed by image acquisition every 10 sec for 10 minutes. Normalized mNeonGreen fluorescence recovery was calculated by subtracting the background noise from the ROI intensity measurement; to normalize for photobleaching due to imaging, this measurement was then divided by a fluorescent control ROI intensity measurement with the background noise subtracted as well.

CRISPR Interference Knockdown

Constructs were designed as described previously [64]. For primer design and workflow, see Table S3.8. Knockdown efficiency was measured using a Tecan Spark plate reader. DCas9-CTRL and DAAP1-depleted cells were pelleted and washed with 1X PBS and then resuspended in 1X PBS. An equal number of dCas9-CTRL or DAAP1-depleted cells were loaded into Tecan

black flat 96-well plates and the volume was adjusted to a total of 150 μ L. Three measurements per sample were averaged and three independent transformations were averaged for total knockdown efficiency, normalized to the control and blanked with 1X PBS.

Attachment Challenge Assay

Microfluidic devices were fabricated using a silicon master, which was created using deep reactive ion etching. The silicon master contained a raised channel that had a rectangular cross-sectional area, with a length of 1 cm, a width of 1 mm, and a height of 115 μ m. A polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning) mold was made using a 10:1 base to curing agent ratio, which was mixed for 5 minutes, degassed for 20 minutes, poured over the master, and placed in an oven for 10 minutes at 110 °C to cure. The mold was peeled away from the silicon master and holes were punched for the inlet and outlet of the channel. Afterwards, inlet and outlet ports were created using a custom aluminum mold with pegs to insert the silicone tubing (0.040 inch ID/ 0.085 inch OD, HelixMark). Degassed 10:1 PDMS was poured into the preheated aluminum mold and cured at 110 °C for 1 hour. The inlet and outlet ports were aligned with holes on the top face of the microfluidic device and stuck together using uncured PDMS and 5 minutes of oven heat. Finally, the bottom face of the microfluidic device and a clean glass slide were plasma treated for 10 seconds and aligned together to create a water-tight seal between the two layers. Trophozoites were chilled for 20 minutes on ice and then loaded into microfluidic devices placed in a GasPak EZ anaerobic pouch (BD Scientific) at 37 °C for 1 h to allow re-attachment. Growth media was drawn into a disposable syringe (BD Scientific) and then loaded onto a syringe pump (Harvard Apparatus). For experiments, the flow rate was set to 20 μ L/min for 30 s to clear swimming trophozoites and then ramped to 100 μ L/min over 5 s and

challenged for 60 s. The challenge assays were recorded at 2 FPS using a Nikon Eclipse Ti microscope with a 40X objective, using phase illumination, Clara DR-1357 CCD camera (Andor), and a live-cell incubation chamber (In Vivo Scientific, Inc) to maintain a temperature of 37 °C. Trophozoite mean speed was quantified starting at 35s using the TrackMate ImageJ plugin via manual tracking over the first 10 seconds of 100 μ L/min flow [29].

TIRF Microscopy

Cells were prepared for live imaging as described above, with the addition of CellMask™ Orange Plasma membrane Stain (ThermoFisher) according to manufacturer's instructions after media replacement with 1X HBS. Cells were imaged in a chamber with 2% CO₂ and 5% O₂ with a DeltaVision OMX (GE) using 60X TIRF objective. Images were taken from randomly selected fields of view. Each dish was imaged for a maximum of 30 minutes to minimize stress on the cells.. Three dishes from two independent experiments were used for each condition.

Live Imaging with Microspheres

Cells expressing DAAP1-mNeonGreen were transfected with a plasmid containing Halo-tagged delta-giardin and either dCas9 control or dCas9+sgRNA 392-411 to deplete DAAP1. Cells were grown in parallel and upon confluency were chilled with ice for 15 minutes to detach from the culture tube and then placed into an Attotfluor cell chamber (Molecular Probes), then incubated in a GasPak EZ anaerobic pouch (BD) or a Tri-gas incubator (Panasonic) set to 2.5% O₂, 5% CO₂ for 90 minutes at 37 °C. 646 HaloTag ligand (Promega, GA1120) was added to cells 45 minutes after incubation at 1:1000 concentration, incubated for 15 minutes, and then replaced with TYDK for 30 minutes prior to imaging. 2.5 μ L of fluorescent microspheres (0.2 μ m,

Polysciences Fluoresbrite® YO Carboxylate Microspheres catalog number 19391) were added to 0.5 mL 1xHBS and sonicated for 10 minutes using a bath sonicator to disrupt aggregates. This diluted solution was then added to cells at a 1:10 ratio to each chamber (30 μ L diluted bead solution in 300 μ L cells). Images of single focal planes in which the disc was visible were taken every 1 second for 15 seconds for each condition. Cells with beads which were collected under their ventral disc and cells with no beads were counted. Cells which detached or moved were excluded from analysis. Live cell imaging was performed on a DeltaVision Elite microscope (GE) equipped with DIC optics, using a 60×1.42 NA objective, and a sCMOS 5.4 PCIe air-cooled camera (PCO-TECH).

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3.1 CHAPTER 3 TABLES AND FIGURES

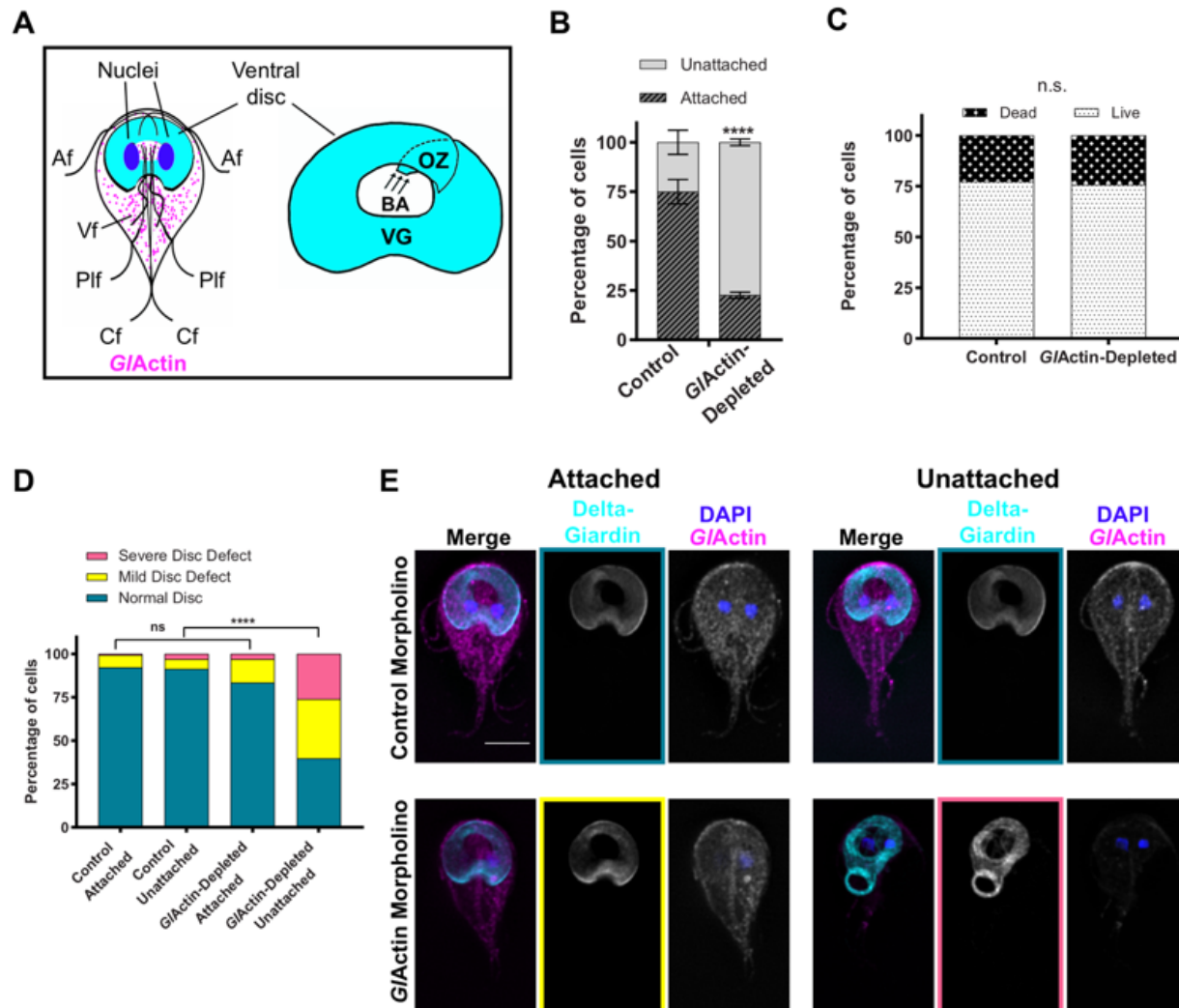


Figure 3.1. Depletion of *G/Actin* affects attachment and ventral disc morphology

A) Left: Diagram of a *Giardia* trophozoite showing *G/Actin* (magenta), disc marker delta-giardin (cyan), and DAPI/nuclei (blue). Anterior (Af), posteriolateral (Plf), ventral (Vf), and caudal flagella (Cf) are shown. Right: Enlarged diagram of ventral disc and areas: overlap zone (OZ), ventral groove (VG), and bare area (BA). Arrows indicate the origin of the microtubule sheet and orientation of the microtubule plus ends. **B)** Quantification of non-challenged attachment assays. Attached and unattached cells were counted 24 hours after treatment with either a standard control or anti-*G/Actin* morpholino. Graph is representative of an average of three replicates, mean $\pm$ sem. For control, 74% $\pm$ 6 were attached and 25% $\pm$ 6 were detached. For *G/Actin*-depleted cells 23% $\pm$ 2 were attached and 77% $\pm$ 2 detached. Raw numbers and detailed statistics reported in Table S3.6. Each replicate was analyzed separately with a two-sided chi-square test with Yates' correction, $P < 0.0001$ for each. **C)** Unattached cells from B were stained with propidium iodide and fluorescein diacetate to distinguish dead from living cells respectively. N=198 control cells and 222 *G/Actin*-depleted cells. There was no significant difference between the two groups based on a two-sided Fisher's exact test $P = 0.7307$. **D)** Both attached and

unattached *GI*Actin-depleted and control cells were fixed and immunostained for delta-giardin to assess ventral disc morphology using fluorescence microscopy. N=126 cells for each condition. There was a significant difference in unattached cells based on a two-sided chi-square test with Yates' correlation, $P < 0.0001$. No significant difference was detected in attached cells based on the same test, $P < 0.0903$ **E)** Representative images of cells from D, with examples of normal discs (teal), mild defects (yellow), and severe defects (pink). Scale bar denotes 5 μm .

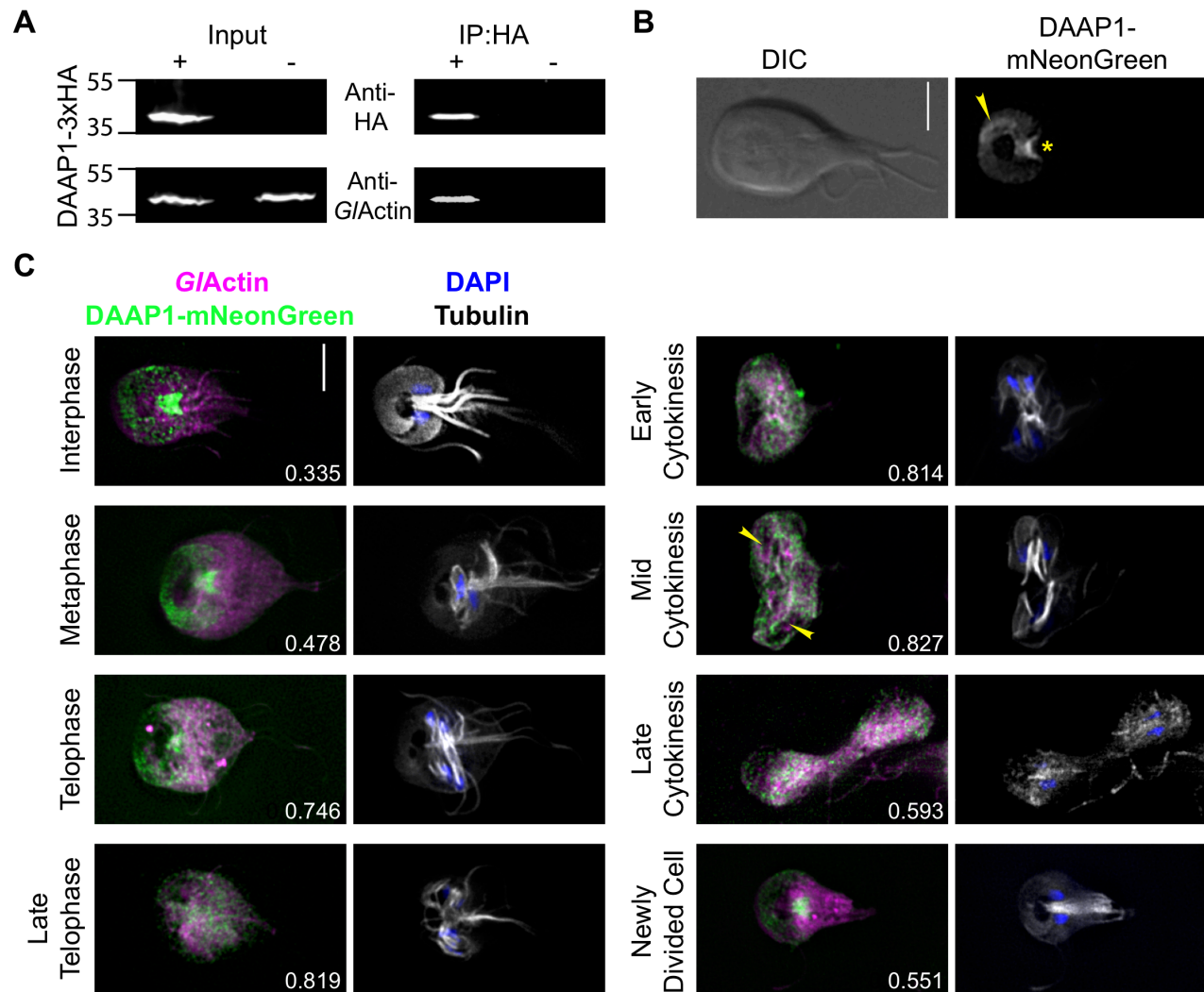


Figure 3.2. DAAP1 is a *G/Actin* interactor and a ventral disc-associated protein

A) Immunoprecipitation from extracts of cells expressing DAAP1-3XHA and wild type cells, followed by western blots probed with anti-*G/Actin* and anti-HA antibodies, revealed interaction between the two proteins. **B)** Image of DAAP1-mNeonGreen in a live cell. The fusion protein localizes to the disc and is enriched in the ventral groove (asterisk) and overlap zone (arrow). Scale bar denotes 5 μ m. **C)** Immunofluorescence of *G/Actin* (magenta), tubulin (cyan), DAAP1-mNeonGreen (green), and DAPI/nuclei (blue) during the cell cycle. DAAP1-mNeonGreen remains on the disc into telophase, is incorporated into the nascent discs by mid cytokinesis (see arrows), and localization is fully restored in newly divided cells. Pearson's correlation coefficients using Costes randomization were calculated for colocalization between DAAP1 and *G/Actin* in each image, shown in the lower righthand corner. The Costes P-value was 1 for all images. Scale bar denotes 5 μ m.

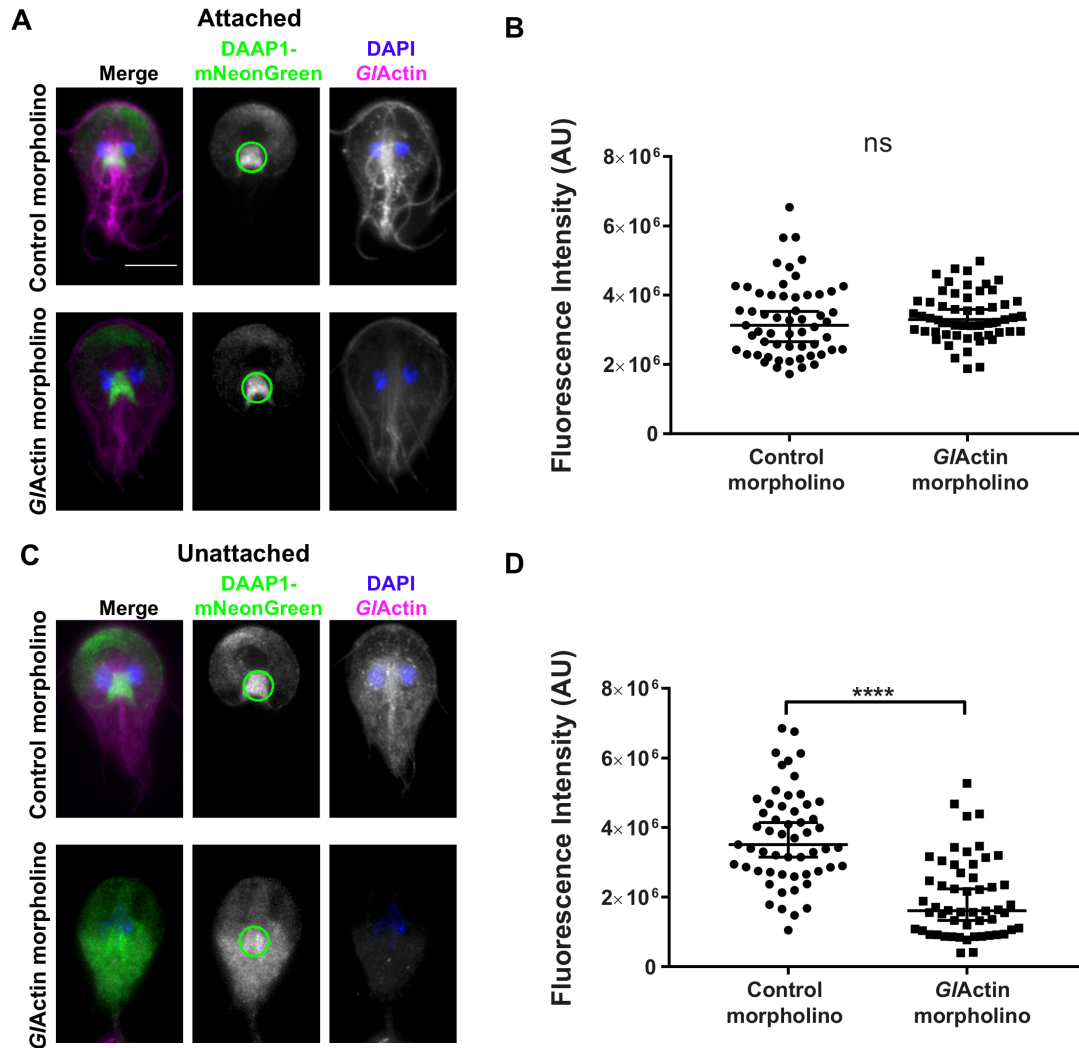


Figure 3.3. DAAP1 loading onto the ventral disc requires *G/Actin*.

A) Representative images of attached cells 24 hours after treatment with either an anti-*G/Actin* or control morpholino. Fluorescence intensity of DAAP1 at the ventral groove (see green circle) was measured at the brightest section in each stack. *G/Actin* shown in magenta, mNeonGreen-DAAP1 in green, DAPI in blue. Scale bar denotes 5 μ m. **B)** A two-tailed Mann-Whitney test indicated that this difference was not statistically significant, ($N_{\text{Control}}=57$, $N_{\text{G/Actin-depleted}}=55$), $P=0.227$. Median and 95% confidence interval shown. Median of control=3131445. Median of *G/Actin*-depleted=3298353. Two and four outliers were detected by Tukey Fence, $k=1.5$ in the control and *G/Actin*-depleted condition respectively and were not included in the analysis. **C)** Representative images of unattached cells 24 hours after treatment with either an anti-*G/Actin* or control morpholino. Fluorescence intensity of DAAP1 at the ventral groove (see green circle) was measured at the brightest optical section in each stack. **D)** A two-tailed Mann-Whitney test indicated that this difference was statistically significant, ($N_{\text{Control}}=55$, $N_{\text{G/Actin-depleted}}=54$), $P<0.0001$. Median and 95% confidence interval shown. Median of control= 3508064. Median of *G/Actin*-depleted=1609904. Four outliers were detected by Tukey Fence, $k=1.5$ in the *G/Actin*-depleted condition and were not included in the analysis.

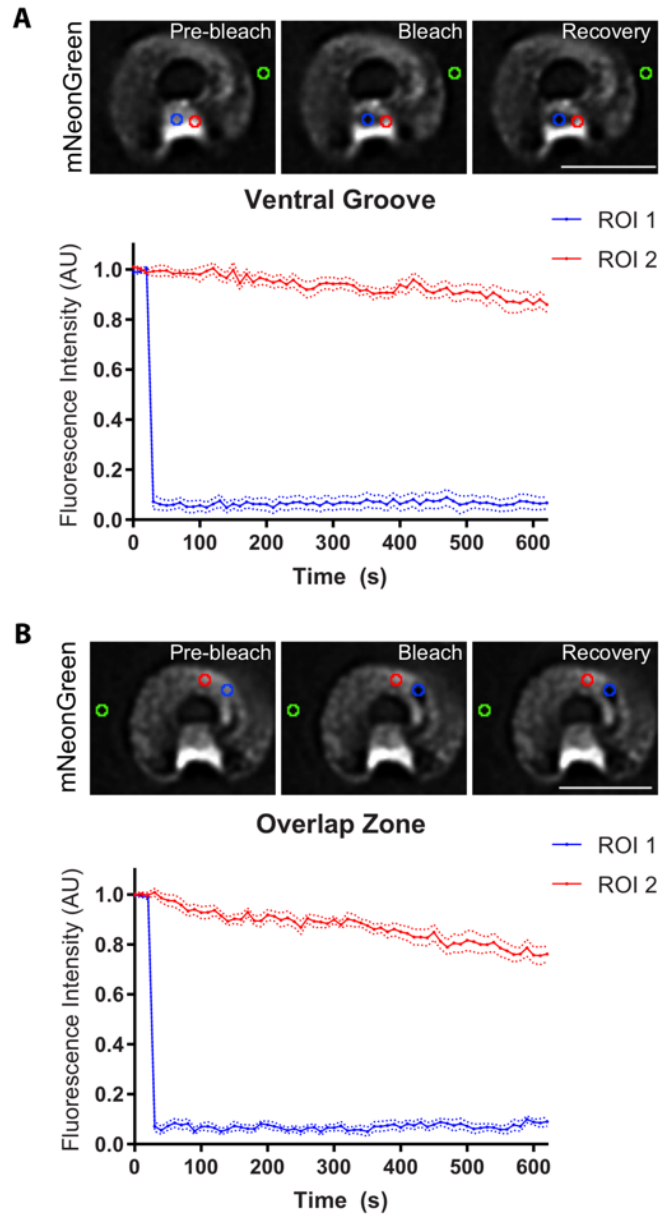


Figure 3.4. DAAP1 is stably associated with the ventral disc

Fluorescence recovery after-photobleaching (FRAP) in DAAP1-mNeonGreen-cells showed no recovery after 12 minutes for bleached areas in both the ventral groove (**A**) and overlap zone (**B**). Three points of reference were taken, including bleached (ROI1, blue), unbleached (ROI2, red), and background (green), and normalized accordingly. Scale bar denotes 5 μm . N=10 for each ROI in both A and B.

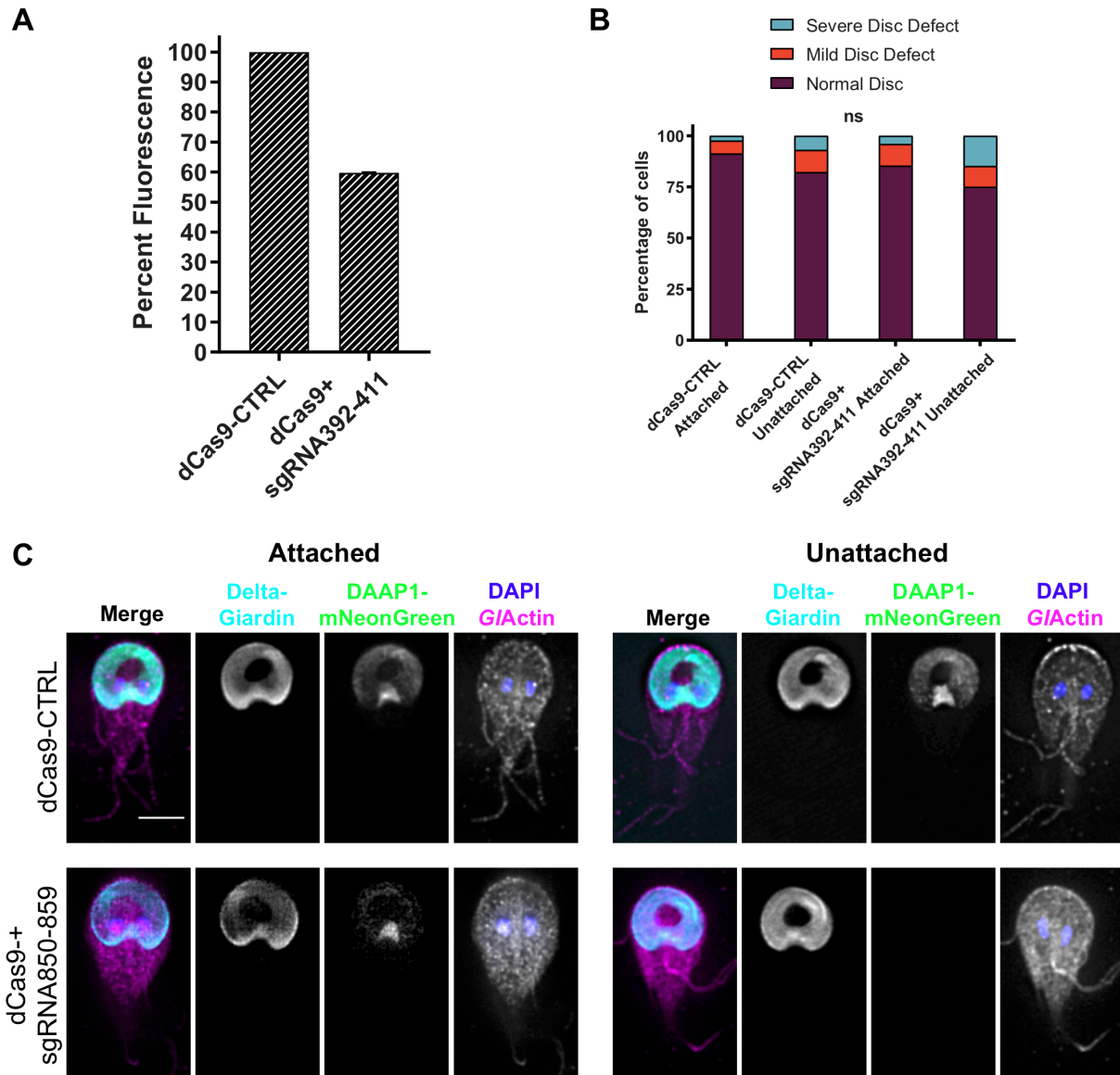


Figure 3.5. DAAP1-depleted cells have no gross disc morphology defects

A.) Fluorescence of DAAP1-mNeonGreen cells transfected with either a non-specific guide RNA (dCas9-CTRL) or a vector containing a small guide RNA targeting DAAP1 (dCas9+sgRNA392-411). Normalized to the control, the DAAP1-depleted cells demonstrated an average knockdown of $40\% \pm 3$ (three independent transformations). Attached and unattached control and DAAP1-depleted cells were fixed and stained separately before assessing their disc morphology. Based on a chi-square test, there was no significant difference in disc defects observed between either attached or unattached groups. (Control attached N=129 cells, control unattached N=126, DAAP1-depleted attached N=123, DAAP1-depleted unattached N=128.) Attached cells, $P=0.3483$. Unattached cells: $P=0.1288$. **C.)** Representative images from B: *Gl*Actin (magenta), delta-giardin (cyan), DAAP1 (green), and DAPI/nuclei (blue). Scale bar denotes 5 μm .

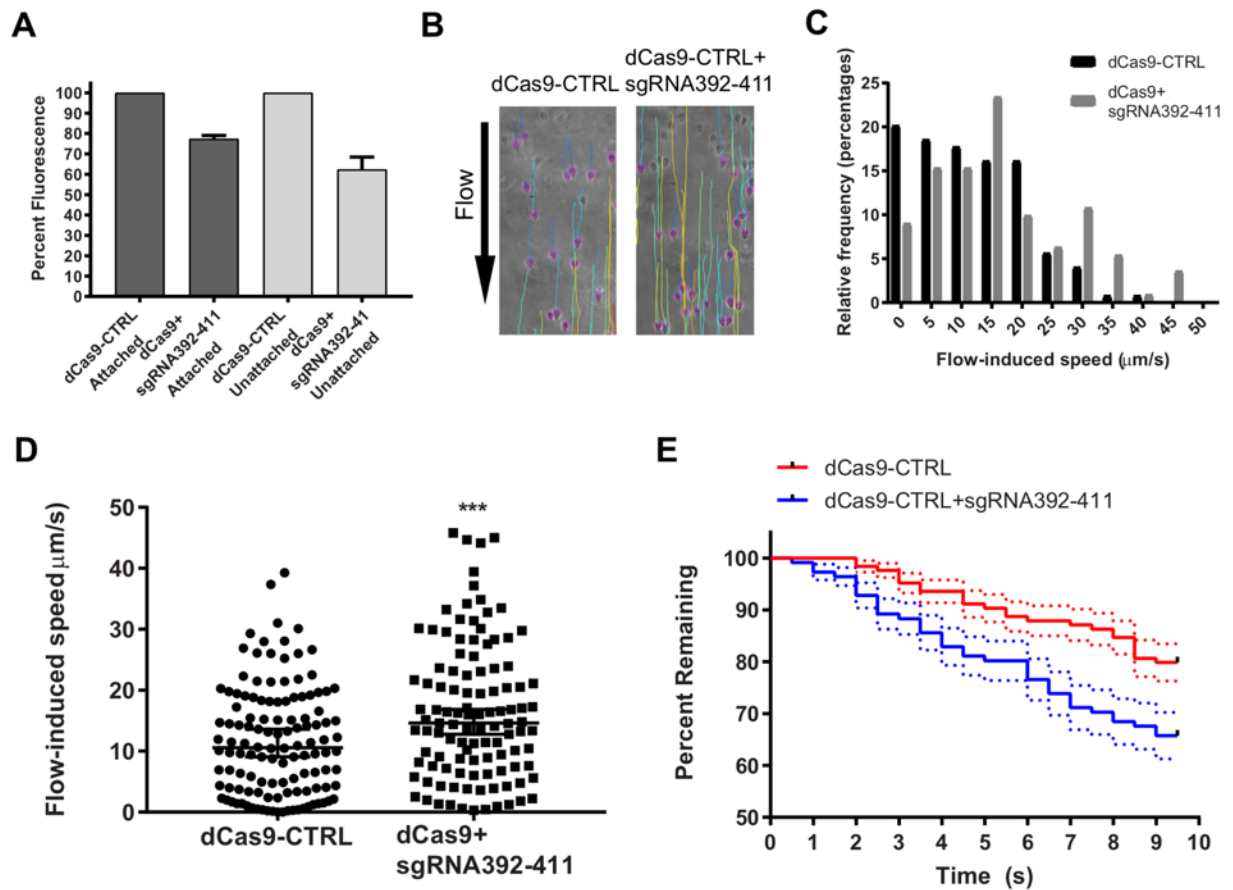


Figure 3.6. DAAP1 depletion results in reduced attachment

A) DAAP1 fluorescence levels from control (dCas9-CTRL) and DAAP1-depleted (dCas9+sgRNA392-411). Unattached cells had a greater decrease in DAAP1 expression ($38\% \pm 6$) than attached cells ($77\% \pm 2$). Average of two independent transformations with three technical replicates for each is shown. **B)** In this flow chamber assay, attached cells were challenged with a flow rate of $100 \mu\text{L}/\text{min}$. Cells were tracked for 10 seconds using TrackMate software in ImageJ, colored lines indicating resulting paths. **C)** Distribution of mean flow-induced speed. **D)** Mean flow-induced speed of each cell for 10 seconds after challenge, with median and 95% confidence intervals. A two-tailed Mann-Whitney test indicated that this difference was statistically significant, ($N_{\text{dCas9-CTRL}}=124$, $N_{\text{dCas9+sgRNA392-411}}=111$), $P=0.001$. Median of control=10.56, upper 95% CI= 13.33, lower 95% CI=10.15. Median of DAAP1-depleted is 14.62, upper 95% CI=18.62, lower 95% CI=14.41. **E)** Kaplan-Meier curve of cells displaced from the field of view over time, $P= 0.0077$ $N=124$ for control, $N=111$ for DAAP1-depleted cells. Median and standard error shown.

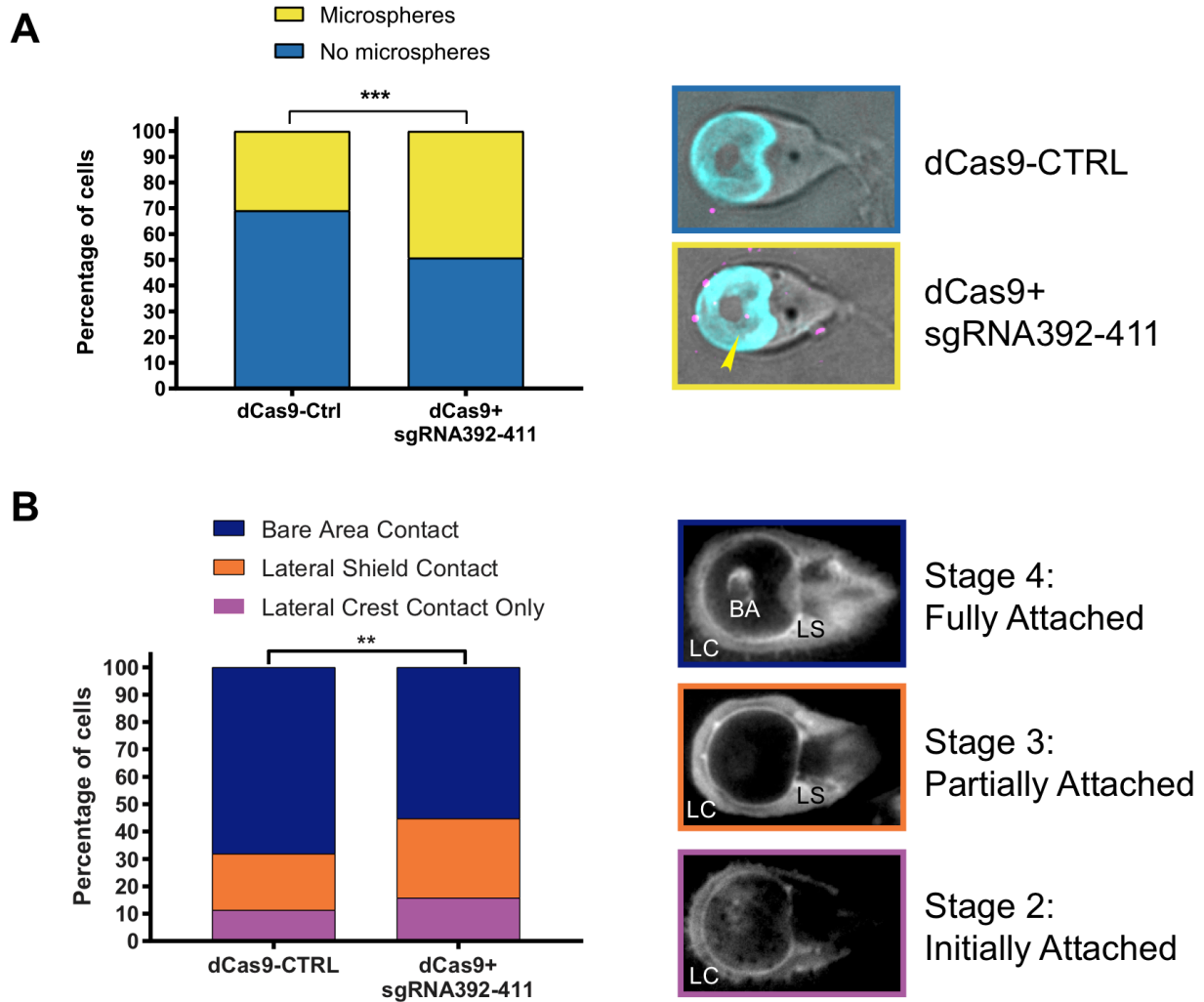


Figure 3.7. DAAP1 has a role in ventral disc seal formation

A.) Fluorescent microspheres were added to live attached control (dCas) and DAAP1-depleted (dCas9+sgRNA392-411) and imaged. Left: significantly more cells with microspheres under the disc were observed in DAAP1-depleted cells based on a chi-square test exact test, $P < 0.001$. $N = 175$ for control, 180 for DAAP1-depleted cells. Right, top: control cell. Right, bottom: DAAP1-depleted cell with microsphere under disc (yellow arrow). Merged images shown with DIC, microspheres (magenta), delta-giardin (cyan). Two-sided Fisher's exact test, $P = 0.0156$ was used for analysis. **B.)** TIRF microscopy assay to monitor the quality of attachment. Control and DAAP1-depleted cells were stained with the plasma membrane stain CellMask Orange and imaged with TIRF microscopy. Left: significantly more DAAP1-depleted cells were partially attached than control cells based on a chi-square test, $N = 317$ for each condition, $P < 0.005$. Right: images of cells in initial attachment (stage 2) with lateral crest (LC) contact, partial attachment (stage 3), with lateral crest and lateral shield (LS) contact, or fully attached (stage 4), with lateral crest, lateral shield, and bare area (BA) contact.

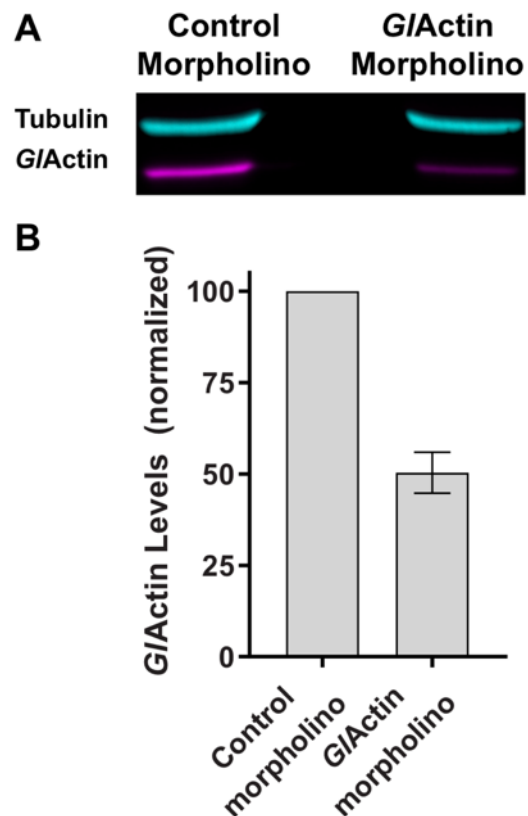


Figure S3.8. *GI*Actin Depletion by antisense translation-blocking Morpholinos

A) Representative western blot of cells treated with either a standard control morpholino (left) or morpholino targeting *GI*Actin (right) for 24-hours and probed for tubulin (cyan) and *GI*Actin (magenta), resulting in a ~61% reduction of *GI*Actin levels. **B)** Average of three morpholino knockdowns results in an average of $50.3\% \pm 5.6$ reduction of protein expression, as determined by western blot. Values shown are mean $\pm$ SEM.

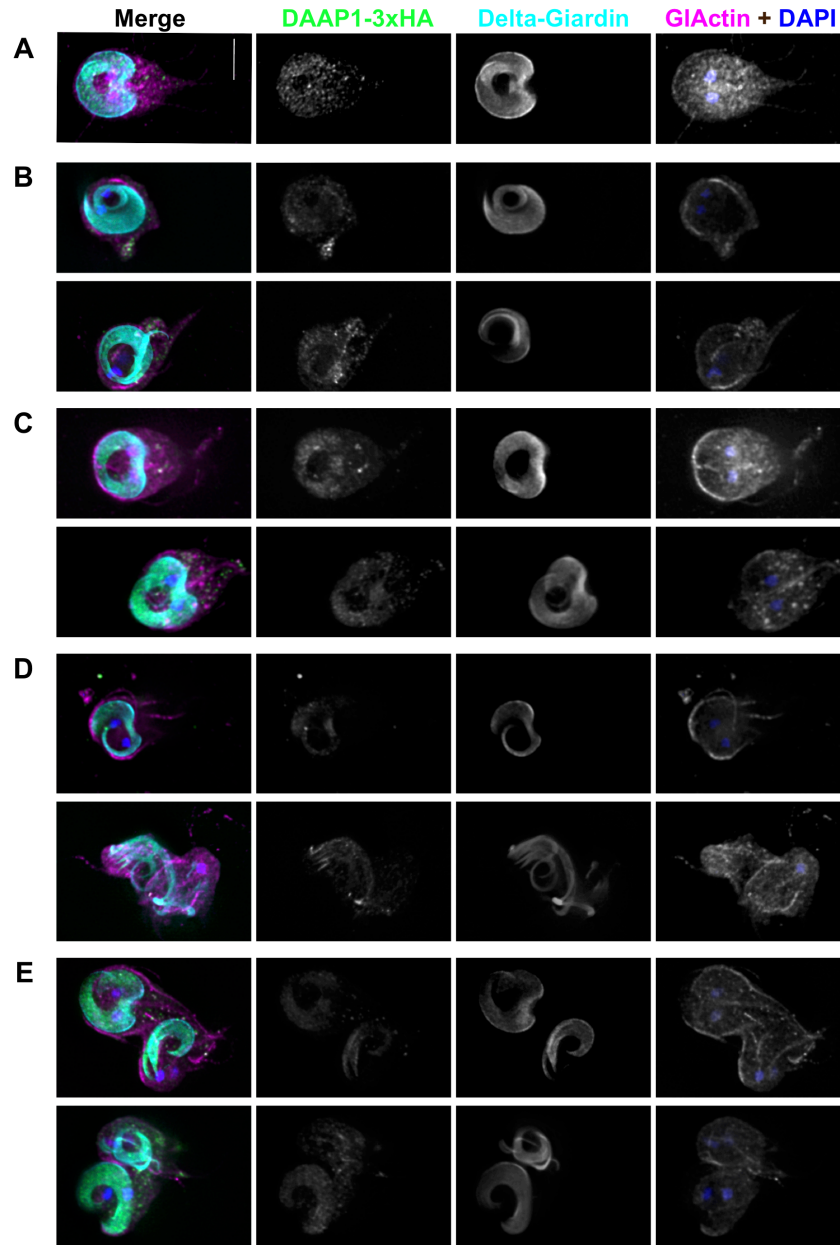


Figure S3.9. Range of Disc Defect Phenotypes in *GIActin*-Depleted Cells

A) Representative *Giardia* cell from the control (attached) condition. (B-E) are *GIActin* depleted cells with disc defects. Mild defects were categorized as discs which were broken (B) or misshapen (C). Severe defects included unwound discs (D) or disc defects associated with failed cytokinesis (E). All cells pictured in B-E were unattached, with the exception of the bottom row of B. Scale bar denotes 5 μ m.

Table S3.6. Raw number of *Gl*Actin-depleted attachment assay cell counts and statistics

Raw numbers			
	Replicate 1	Replicate 2	Replicate 3
Control Attached	2000000	1750000	891000
Control Unattached	302000	884000	353000
Actin-Depleted Attached	487000	403000	144000
Actin-Depleted Unattached	1480000	1671500	466000
Percentages			
Control	86.8809731	66.4388762	71.62379421
Control Unattached	13.1190269	33.5611238	28.37620579
Actin-Depleted Attached	24.7585155	19.4263678	23.60655738
Actin-Depleted Unattached	75.2414845	80.5736322	76.39344262
Two-sided Chi-square test statistics with Yates' correction			
Replicate 1	X2 (1, N=4269000)=1683251, P<.0001.		
Replicate 2	X2 (1, N=4708500)=991308, P<.0001.		
Replicate 3	X2 (1, N=1854000)=382673, P<.0001.		

Table S3.7. DAAP1-depleted attachment assay cell counts and statistics

Replicate 1				
Unattached	Read			
	1	2	Total	Percent
dCas9-CTRL	3.10E+05	3.04E+05	614000	26%
dCas9+sgRNA392-411	3.18E+05	3.10E+05	628000	27%
Attached	Read			
	1	2	Total	Percent
dCas9-CTRL	8.67E+05	8.70E+05	1737000	74%
dCas9+sgRNA392-411	8.56E+05	8.50E+05	1706000	73%
Total:				
dCas9-CTRL	2.35E+06			
dCas9+sgRNA392-411	2.33E+06			
X2 (1, N = 4685000) = 155584079137.67, p <.00001				
Replicate 2				
Unattached	Read			
	1	2	Total	Percent
dCas9-CTRL	1.23E+05	1.19E+05	2.42E+05	13%
dCas9+sgRNA392-411	1.66E+05	1.68E+05	3.34E+05	16%
Attached	Read			
	1	2	Total	Percent
dCas9-CTRL	7.94E+05	7.88E+05	1.58E+06	87%
dCas9+sgRNA392-411	8.90E+05	8.78E+05	1.77E+06	84%
Total:				
dCas9-CTRL	1.82E+06			
dCas9+sgRNA392-411	2.10E+06			
X2 (1, N =3926000) = 5363.32, p <.00001				
Replicate 3				
Unattached	Read			
	1	2	Total	Percent
dCas9-CTRL	1.37E+05	1.40E+05	2.77E+05	16%
dCas9+sgRNA392-411	1.44E+05	1.45E+05	2.89E+05	16%
Attached	Read			
	1	2	Total	Percent
dCas9-CTRL	7.36E+05	6.99E+05	1.44E+06	84%
dCas9+sgRNA392-411	7.56E+05	7.42E+05	1.50E+06	84%
Total:				
dCas9-CTRL	1.71E+06			
dCas9+sgRNA392-411	1.79E+06			
X2 (1, N = 3499000) = 0.037, p =.849				

Table S3.8. Primer and morpholino sequences and workflow

Gene	Plasmid	Primer Sequences	Episomal or Integrated	Notes
GL50803_16844	pks_16844_mNeon_Neo	F- 5' ctccaccgcggtggcgccgcCTAC AGATCCCGTTAAACC 3' R-5' cctgaaccgcctccggatccATAAA CAGAGACGTTTGCTAAC 3'	Integrated	Construct with C-terminal tag, 16844 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes NotI-HF and BamHI-HF were used to digest pks mNeonGreen N11 NEO vector; Gibson reaction was used for assembly. Aval was used for linearization
GL50803_16844	pks_16844_3HA_Neo	F- 5' gcaggaaatcgatatcaagcttCTACA GATCCCGTTAAACC 3' R-5' cgtcataaggatattccttaagATAAA CAGAGACGTTTGCTAAC 3'	Integrated	Construct with C-terminal tag, 16844 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. Restriction enzymes HindIII-HF and AflII were used to digest pks 3HA NEO vector; Gibson reaction was used for assembly. XhoI was used for linearization
GL50803_86676	pks_HALO_86676_PAC	F-5' ccgctctagaactagtggatccAATG GACACAAGAGATGC 3' R-5' agcaacgatgatgcaagaattcTTAA CCGGAATCTCCAG 3' F-5' tttgtcttcCTCGAGCCAACCAC TGAG 3' R-5' aaaggctcgacggaagaattcTTAAC CGGAATCTCCAG 3'	Episomal	Construct with C-terminal tag, 86676 coding sequence plus 200 base pairs of upstream sequence were amplified from WBC6 genomic DNA. HALO tag was amplified from HALO vector. Restriction enzymes EcoRI-HF and NotI-HF were used to digest pks 3XHA PAC vector; All three fragments were assembled using In-Fusion cloning reaction (Takara Bio).
GL50803_16844 CRISPRi Construct	dCas9_sgR NA392-411 PAC	F- 5' CAAAGCACGACCCTACCA TGGCCA 3' R- 5' AAACTGGCCATGGTAGGG TCGTGC 3'	Episomal	dCas9 PAC plasmid was digested with BbsI. Plasmid was assembled with T4 Ligase.
GL50803_16844 CRISPRi Construct with 86676 HALO	dCas9_PAC and dCas9_sgR NA392-411 PAC	F-5' tatagaataactcaagcttggACATCC TGCACCGTAGTG 3' R-5' agatcaacttactaataggTAAACC GGAAATCTCCAGAG 3'	Episomal	86676-HALO was amplified from above plasmid and dCas9 plasmid, either empty or with sgRNA, was digested with AscI. Plasmids were assembled with Gibson cloning.
Morpholino sequences				
Gene target	Sequence			
N/A (Standard control)	CCTCTTACCTCAGTTACAATTTATA			
GL50803_40817 (GlActin)	GCAGGGTTGTCGTCTGTCATTTTAC			
GL50803_16844	AAAAGTTTCTTCCGCGCAAAGGCC			

Table S3.9. Number of microspheres per cell and statistics

Number of microspheres per cell	
dCas9-CTRL	dCas9+sgRNA392- 411
0	0
0	0
0	0
0	0
0	0
0	0
0	0
0	0
0	0
0	0
0	0
0	0
0	0
0	0
0	0
0	0
0	0
0	0
0	0
0	1
0	1
0	1
0	0
0	4
0	0
0	0
0	0
0	0
0	0
0	2
0	0
0	0
0	0
0	0
0	1
0	1
0	1
0	0
0	0
0	0
0	0
0	0
0	2
0	0
0	0
0	0
0	

0	
0	
0	
0	
0	
0	
0	
0	
0	
1	
0	
0	
0	
0	
1	
0	
1	
0	
0	
0	
0	
0	
2	
0	
0	
0	

A Mann-Whitney test, indicated that this difference was statistically significant
 $U(N_{\text{dCas9-CTRL}}=65, N_{\text{dCas9+392-411}}=39)=1.51,$
 $P=.0101$

3.2 CHAPTER 3 REFERENCES

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